H$_2$S-releasing drugs: Anti-inflammatory, cytoprotective and chemopreventative potential

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**ABSTRACT**

Hydrogen sulfide exerts a number of cytoprotective and anti-inflammatory effects in many organ systems. In an effort to exploit these potent and beneficial effects, a number of hydrogen sulfide-releasing derivatives of existing drugs have been developed and extensively tested in pre-clinical models. In particular, efforts have been made by several groups to develop hydrogen sulfide-releasing derivatives of a number of nonsteroidal anti-inflammatory drugs. The main goal of this approach is to reduce the gastrointestinal ulceration and bleeding caused by this class of drugs, particularly when used chronically such as in the treatment of arthritis. However, these drugs may also have utility for prevention of various types of cancer. This paper provides an overview of some of the mechanisms underlying the anti-inflammatory and cytoprotective actions of hydrogen sulfide. It also gives some examples of hydrogen sulfide-releasing anti-inflammatory drugs, and their actions in terms of reducing inflammation and attenuating the development of cancer in experimental models.

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**1. Introduction**

Over the past decade, several novel H$_2$S-based drugs have been described [1,2]. Several of the most advanced such compounds are derivatives of currently used anti-inflammatory drugs. The H$_2$S-releasing drugs show greatly reduced toxicity (particularly in the GI tract) and, in some cases, improved efficacy. Several nonsteroidal anti-inflammatory drugs (NSAIDs) have been found to substantially reduce the incidence of several types of human cancers, but their adverse effects preclude their use for these indications, which would require long-term exposure to the drugs. H$_2$S itself exerts potent anti-inflammatory effects [3] and, in some test systems, anti-cancer effects [4,5]. In this article, we describe some of the features of H$_2$S-releasing drugs that we have developed with respect...
to their potential use as anti-inflammatory, analgesic and chemoprevention applications, as well as discussing the cytoprotective mechanisms of these drugs that so profoundly reduce their toxicity.

2. Anti-inflammatory and pro-resolution effects of hydrogen sulfide

Among the earliest events in an acute inflammatory reaction is the adherence of leukocytes to the vascular endothelium and their subsequent extravasation and migration to sites of injury. A key early step in this process is up-regulation of adhesion molecules on leukocytes and on the vascular endothelium. H₂S plays a very important role in regulating these processes [Fig. 1]. In fact, tonic production of H₂S is an important contributor to the down-regulation of leukocyte adhesion in the healthy state. When an inhibitor of H₂S synthesis is administered, there is a rapid increase in leukocyte adherence to the vascular endothelium [3]. This process is mediated via up-regulation of lymphocyte function-associated antigen (LFA) on circulating leukocytes and P-selectin and intercellular adhesion molecule (ICAM-1) on the endothelium [6] (Fig. 1). Rats with a diet-induced vitamin B deficiency, which have a diminished capacity to synthesise H₂S, also exhibit significantly enhanced accumulation of leukocytes in the affected tissues [7]. Mice that are heterozygous for the cystathionine β-synthase gene also have diminished synthesis of H₂S, and they exhibit increased levels of leukocyte adherence to the vascular endothelium, slower leukocyte rolling velocity and increased vascular permeability [8].

In contrast, a reduction of leukocyte adherence can be seen after administration of H₂S donors [3]. This effect appears to be mediated by activation of adenosine triphosphate (ATP)-sensitive potassium channels on endothelial cells and leukocytes. H₂S donors have also been shown to inhibit endothelial ICAM-1 expression triggered by high blood-glucose concentrations [9]. H₂S donors have been shown to cause a marked suppression of inflammatory responses in several animal models, including colitis and endotoxic shock [10,11].

In addition to reducing leukocyte extravasation and migration to sites of injury, H₂S can reduce the cytotoxic effects of neutrophils by inhibiting myeloperoxidase activity [12]. Moreover, H₂S promotes apoptosis of neutrophils, a key step in resolution of inflammation [13]. H₂S also increases phagocytosis of bacteria by macrophages, and promotes a shift of phenotype of macrophages to the “M2”, pro-resolution state [14]. H₂S donors can also reduce inflammation by reducing the expression of a number of pro-inflammatory cytokines (e.g., tumour necrosis factor (TNF)-α, interleukin (IL)-1β, IL-8, interferon (IFN)-γ but sparing of expression of the pro-inflammatory cytokine, IL-10 [10,11,15]. Inhibition of pro-inflammatory cytokine expression most likely occurs by suppression of nuclear factor-κB (NF-κB) activity by H₂S [16]. We recently described a regulatory interaction between IL-10 and H₂S synthesis in the colon (and these mice can spontaneously develop colitis) [7,17]. Administration of H₂S donors increases IL-10 expression [7,15], while administration of IL-10 to the IL-10-deficient mice restored normal colonic H₂S synthesis [7].

Some of the resolution-promoting effects of H₂S are mediated via induction of cyclooxygenase (COX)-2 expression, and the ensuing synthesis of anti-inflammatory eicosanoids such as prostaglandin (PG) E₂ [18,19]. In the GI tract, for example, inhibition of H₂S synthesis leads to a decrease in COX-2 expression, a decrease in PGE₂ synthesis, an increase in mucosal inflammation and an impairment of healing of damaged tissue [18–21]. Administration of H₂S donors has the opposite effects [20–22]. The augmentation of COX-2 expression by H₂S donors likely also contributes to their ability to accelerate healing of wounds and ulcers [20,23].

3. Cytoprotective actions of hydrogen sulfide in GI tract

The ability of H₂S to reduce damage in the GI tract is striking, particularly since this cytoprotective effect extends to prevention of the severe injury and bleeding that can be induced in the small intestine by repeated administration of NSAIDs [6,11,22–26]. There are a number of mechanisms of action through which endogenous cytoprotective substances (e.g., PGs, nitric oxide) produce their beneficial effects [27] and considerable data have been generated in recent years with respect to the actions of H₂S in this regard (Fig. 2). For example, H₂S will stimulate secretion of bicarbonate in the stomach and duodenum, thereby reducing the potentially damaging effects of gastric acid [24,28]. H₂S may also directly inhibit gastric acid secretion [29]. A reduction in gastric blood flow is a common feature in the early stages of gastric injury, and it can be prevented by H₂S donors [6]. Likewise, gastric damage induced by NSAIDs is largely mediated, particularly at the earliest stages, by leukocyte adhesion to the vascular endothelium in the gastric microcirculation [30].

As mentioned earlier, the ulceration and bleeding caused by NSAIDs in the small intestine is much more complex, in terms of pathogenesis, and develops over a longer period of time than the damage in the stomach [31,32]. Unlike damage in the stomach and duodenum, gastric acid does not play a significant role in the damage induced by NSAIDs in the more distal small intestine. Indeed, the use of proton pump inhibitors and histamine H₂-receptor antagonists to reduce NSAID-induced damage in the upper GI tract results in exacerbation of ulceration and bleeding in the small intestine [33,34]. This is a consequence of significant changes in the

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The mechanisms through which H$_2$S can protect against NSAID-enteropathy appear to include changes in at least three of the key factors that are central to the pathogenesis of this injury: the composition/secretion of bile, the microbiota and the enterohpatic recirculation of NSAIDs [22]. Thus, H$_2$S reduces the cytotoxicity of bile (which is elevated by NSAIDs), promotes a normalisation of the microbiota (which is altered by NSAIDs) and reduces the enterohpatic circulation of NSAIDs and/or NSAID-glucuronides [22,24,35].

4. GI-sparing anti-inflammatory drugs

NSAIDs are among the most commonly used drugs, because they effectively reduce pain and inflammation. The use of this class of drugs on a chronic basis is for joint inflammation, most notably osteoarthritis. While very effective, and non-addictive, NSAIDs do carry a significant risk for adverse effects, with the most clinically significant being GI ulceration and bleeding. Unfortunately, selective COX-2 inhibitors provided only a partial solution to this problem, and their use is associated with significant cardiovascular toxicity [36]. H$_2$S appears to provide a solution to these problems. A range of NSAID derivatives that release H$_2$S have been synthesised and evaluated, with consistent results in terms of retaining anti-inflammatory activity but reducing GI toxicity. For example, we have synthesised derivatives of aspirin, naproxen, ketoprofen, indomethacin, ibuprofen, flurbiprofen and diclofenac, each with a number of different H$_2$S-releasing moieties. Data are presented in this paper on aspirin, naproxen and ketoprofen derivatives, as representative examples. The major focus of our research has been on a compound called ATB-346, which consists of naproxen covalently linked to 4-hydroxythiobenzamide (TBZ) [26,34]. We have been studying this drug as a potential treatment for arthritis, a chronic use. Figure 3 shows the effects of ATB-346 and naproxen in a rat model in which arthritis is induced by injection of Freund's adjuvant [37]. ATB-346 produced a dose-dependent reduction in paw oedema in this model, not significantly different from the effects of equinolar doses of naproxen. GI damage was frequently observed in the naproxen-treated rats, but not in the rats treated with ATB-346. Indeed, at the highest dose tested, all of the 12 rats treated with naproxen had died before completion of the study because of perforation of the small intestine (Fig. 3).

We chose naproxen as the NSAID to use as the base drug because it is the only marketed NSAID that has not been associated with significant and serious cardiovascular adverse events [36]. Indeed, concerns about these cardiovascular events have led to several large physician's groups (e.g., American Heart Association, American College of Gastroenterology) to recommend use of naproxen, or of a selective COX-2 inhibitor plus low-dose aspirin (the latter for cardioprotection). However, co-administration of low-dose aspirin with NSAIDs (including selective COX-2 inhibitors) greatly increases the risk of GI ulceration and bleeding [38]. A further recommendation in many patients is that they take drugs to suppress gastric acid secretion (e.g., proton pump inhibitors, histamine H$_2$ receptor antagonists) to protect their stomach from damage, but these drugs significantly worsen ulceration and bleeding in the small intestine [33], where it is more difficult to detect and for which there are no proven-effective preventative or curative treatments [31,32,39,40].

The metabolism of ATB-346 and the mechanism for H$_2$S release from this drug are not completely understood. Release of H$_2$S from ATB-346 has been demonstrated in vitro [41]. The release of H$_2$S from this compound occurs at a very low level when the drug is dissolved in a buffer, but is greatly increased in the presence of tissue or in the presence of reducing agents such as diithiothreitol, L-cysteine.

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or glutathione. One likely scenario of metabolism of ATB-346 is shown in Fig. 4. Hydrolysis of the thiol moiety results in H$_2$S release, leaving naproxen-4-hydroxybenzamide. This compound suppresses COX activity and induces gastric damage when given to rats (Figs 4, 5). As shown in Fig. 5, the rise in plasma naproxen levels in rats after oral administration of ATB-346 is quite slow as compared to that observed after oral administration of naproxen itself. However, there is significant suppression of gastric prostaglandin synthesis within 15 min of administration of ATB-346, when plasma levels of naproxen are very low. This supports the notion that an intermediate with COX-inhibitory activity is rapidly formed after oral administration of ATB-346. The lack of gastric damage observed following oral administration of ATB-346 (Fig. 4) is most likely due to the rapid release of H$_2$S. We have confirmed that H$_2$S donors can replicate this protective effect, and that protection of the small intestine from injury is also achieved [18,22]. The latter is significant because the enteropathy caused by NSAIDs is much more slow to develop than the gastric damage, so one might predict that if H$_2$S was rapidly released from ATB-346, any beneficial effects might not be long-lasting enough to protect the small intestine. Our pharmacokinetic studies demonstrate that the total exposure of ATB-346-derived naproxen from oral dosing with this drug, as compared to dosing with naproxen itself at an equimolar dose, is very similar (~14% lower bioavailability with ATB-346). We have also observed that there is significantly reduced enterohepatic recirculation of naproxen following administration of ATB-346. Biliary levels of naproxen and naproxen–glucuronides were markedly reduced following ATB-346 administration to rats, as compared to rats treated with naproxen itself [34]. It is possible that this effect is also related to alterations in the microbiota, given that bacterial enzymes account for deglucuronidation of NSAID–glucuronides in the intestine [42].

The lack of GI injury in rats treated with ATB-346 is impressive, but there are NSAIDs marketed for treatment of arthritis that are much more toxic in the GI tract than naproxen, and these drugs have particular utility for treatment of acute, severe pain (postsurgery, gout, etc.). Ketoprofen is one such NSAID. We examined the GI-sparing effects of ATB-352, a H$_2$S-releasing derivative of ketoprofen, in rats (6 per group). Oral administration of ketoprofen

**Fig. 4.** Proposed mechanism of release of hydrogen sulfide (H$_2$S) and naproxen from ATB-346 after oral administration. Biological effects consistent with H$_2$S release are evident within minutes of administration of ATB-346 (e.g., gastric vasodilation). While suppression of gastric prostaglandin synthesis and whole blood thromboxane synthesis can be observed within 15 minutes of administration of ATB-346, a significant rise in blood levels of naproxen only occurs over several hours.

**Fig. 5.** Pharmacokinetics and pharmacodynamics of ATB-346. Inhibition of gastric prostaglandin synthesis (and whole blood thromboxane synthesis; not shown) is apparent within 15 min of oral administration of ATB-346 to rats (panel A), but blood levels of naproxen are very low (panel B), suggesting that ATB-346 itself or a rapidly generated metabolite can inhibit cyclooxygenase activity. Release of hydrogen sulfide (H$_2$S) from ATB-346 via hydrolysis results in the formation of naproxen-4-hydroxybenzamide, which can inhibit cyclooxygenase. When administered to rats, naproxen-4-hydroxybenzamide produced significant gastric hemorrhagic damage (panel C), while ATB-346 administration did not induce gastric damage (panel D). Results in panels A and B are shown as the mean ± SEM, with 6 rats per group.
at a dose of 30 mg/kg to fasted rats resulted, within a few hours, in the formation of extensive haemorrhagic erosions in the stomach. The mean gastric damage score was 41 ± 3. In contrast, oral administration of an equimolar dose of ATB-352 did not produce any gastric damage (confirmed by histology). As has been observed with other H2S-releasing NSAIDs, ATB-352 inhibited gastric PGE2 synthesis as effectively as ketoprofen.

To determine if similar safety of ATB-352 would be observed in the small intestine, groups of 6 rats each were treated orally with ketoprofen (20 mg/kg) or an equimolar dose of ATB-352 twice daily for 5 days (the rats were not fasted). Treatment with ketoprofen elicited extensive ulceration and bleeding in the intestine, with a mean damage score of over 700 (Fig. 6). This level of damage is approximately 7 times that observed with naproxen administered at 20 mg/kg. In sharp contrast, administration of an equimolar dose of ATB-352 did not provoke significant intestinal damage (mean score of 2; same as in vehicle-treated rats), despite inhibiting small intestinal PGE2 synthesis and whole blood thromboxane synthesis as effectively as ketoprofen (by >90%; Fig. 6).

Chronic use of aspirin has been shown to reduce the incidence of a range of serious cardiovascular events, as well as reducing the incidence of several types of cancer [43–45]. The primary limitation to such use is the significant risk of aspirin-induced bleeding, particularly in the GI tract. A compound that showed the desirable effects of aspirin without the GI adverse effects would have tremendous potential for chronic use as a chemopreventive agent. With this in mind, we evaluated the properties of a novel H2S-releasing derivative of aspirin, ATB-340. The beneficial effects of aspirin with respect to cardiovascular disease prevention are attributable to the ability of this drug to irreversibly inhibit COX-1, particularly in platelets. Thus, we first determined if ATB-340 exhibited comparable inhibitory activity on COX-1 activity in an in vitro model. Human blood samples were added to tubes containing vehicle, aspirin or ATB-340 at concentrations of 1–30 μM. The blood was allowed to clot at room temperature for 45 min, after which the tubes were centrifuged and the serum was collected for measurement of thromboxane B2 levels by enzyme-linked immunosorbent assay [46]. Both aspirin and ATB-340 inhibited thromboxane synthesis in a concentration-dependent manner (>95% inhibition at 30 μM), with no significant differences observed between the two drugs. We then proceeded to evaluate ATB-340 in vivo. Groups of rats (n = 6 each) were fasted overnight and then given vehicle or one of the two test drugs orally, at doses ranging from 3 to 100 mg/kg. Three hours later, the rats were euthanised and the stomach was examined by an observer blind as to the treatments the rats had received. Haemorrhagic gastric damage was scored (the area of each lesion was measured in mm2 and these were summed to give the total area of all lesions in the stomach). Aspirin caused haemorrhagic damage that increased in severity with dose, while damage was not seen in the rats treated with ATB-340 at equimolar doses. Thus, the level of damage in rats treated with ATB-340 did not differ significantly from that in vehicle-treated rats. We then examined the effects of repeated administration of aspirin vs. ATB-340 in rats. As shown in Fig. 7, administration of aspirin each day for a week at a dose of 10 mg/kg resulted in significant damage to the stomach. In contrast, with daily administration of ATB-340, no significant gastric damage was observed (Fig. 7).

5. Chemopreventative actions of H2S-releasing NSAIDs

There is considerable evidence that regular use of NSAIDs, including aspirin, can significantly reduce the incidence of a number of types of cancer [47,48], and they appear to be particularly effective at reducing the incidence of colon cancer [43,44]. However, their propensity to cause ulceration and bleeding is a major barrier to their use for chemoprevention of cancers (or for other chemoprevention uses). We and others have explored the possibility that GI-sparing, H2S-releasing NSAIDs would produce the beneficial chemopreventative effects without the GI toxicity [49,50]. For example, we evaluated the effects of a H2S-releasing ketoprofen derivative, ATB-352, in a model of pre-cancerous lesions in mice. Five-week-old mice were given injections of azoxymethane (10 mg/kg, i.p.) at weekly intervals (total of 4 injections). The mice were
euthanised 1 week after the final injection of azoxymethane. A laparotomy was performed and the entire colon was excised. After gentle flushing with 0.9% saline, the colon was tied at both ends with silk sutures and insufflated with 10% phosphate-buffered formalin. The colon was submerged in formalin for 24 h, then stained with 0.2% methylene blue. Using a dissection microscope at 40x magnification, the number of aberrant crypt foci (ACF) in the entire colon was blindly determined. ACFs are pre-cancerous lesions which were distinguishable from the surrounding normal crypts by their increased size, significantly increased distance from the lamina to basal surface, and easily discernible pericryptal zone [51]. Quantification of ACF was performed blindly, according to previously published criteria [52].

Groups of at least 6 rats each were treated once-daily for the first 2 weeks of azoxymethane administration with vehicle, ketoprofen at 10 mg/kg, or equimolar doses of ATB-352 or of the H$_2$S-releasing moiety of ATB-352 (TBZ). As shown in Fig. 8, vehicle-treated rats developed an average of ~35 aberrant crypt foci. Treatment with ketoprofen or ATB-352 significantly reduced this number by 40–50%, with no significant difference between these two drugs. The two drugs also inhibited colonic PGE$_2$ synthesis to a similar extent (~90% inhibition). Treatment with TBZ had no significant effect on numbers of aberrant crypt foci, and did not significantly affect colonic PGE$_2$ synthesis.

We further evaluated the chemopreventative effects of ATB-352 in mice with a genetic defect that predisposes them to intestinal cancer [53]. Male Apc$^{Min^+}$ mice were obtained from Jackson Laboratories (Bar Harbor, MA, USA). The mice were treated with vehicle, ketoprofen at 10 mg/kg, or equimolar doses of ATB-352 or TBZ once-daily for 2 weeks (beginning at 6 weeks of age). When the mice reached week 14 of age, the colon and small intestine were examined for polyps. The area (in mm$^2$) of polyps was measured under a dissecting microscope as described previously, by an observer unaware of the treatments the mice had received [54]. The total area of all polyps was calculated as the “polyp score”.

Figure 9 shows the effects of the drugs on the polyp score. In vehicle-treated mice, the average polyp score was ~100. Treatment with ketoprofen significantly reduced the polyp score by ~50% ($p < 0.05$). Treatment with ATB-352 was significantly more effective than treatment with ketoprofen (~60% reduction). However, TBZ had no significant effect.

These results in animal models suggest that H$_2$S-releasing NSAIDs may be effective and safe in chemoprevention of colon cancer, and other types of cancers. The mechanism of action of these drugs is not clear, but suggested mechanisms for the anti-cancer actions of H$_2$S include inactivation of Nrf2 (nuclear factor erythroid 2-related factor 2) via sulphydratation of Keap1 [55], induction of glycolysis within cancer cells [5] and induction of apoptosis of cancer cells [56]. Inhibition of COX-2 activity could certainly be a contributing effect to the reduction of polyps and tumours, but the enhanced activity of ATB-352 as compared to ketoprofen is unlikely due to different effects on COX-2 activity. Our data demonstrated that these two drugs inhibit colonic PGE$_2$ synthesis to the same extent.

6. Future directions

Proof-of-concept animal studies of H$_2$S-releasing anti-inflammatory drugs are very encouraging, particularly as such consistent effects have been observed across a range of anti-inflammatory drugs and a range of H$_2$S-releasing moieties. These compounds exhibit profoundly increased safety profiles, particularly with respect to GI damage. They also often exhibit enhanced anti-inflammatory activity. NSAIDs are used primarily for treating acute and chronic pain, but as discussed in this paper, they are potentially very useful for a number of other indications, such as chemoprevention of cancer. The toxicity of NSAIDs is the major barrier to their widespread use in chemoprevention. Thus, the marked reduction of toxicity through linking of NSAIDs to H$_2$S-releasing moieties opens up the possibility for much wider and longer-term use of these drugs.

Clinical studies of several H$_2$S-releasing drugs are now underway. With the advancement of such drugs into human studies, the need for improved methods for detection and measurement of H$_2$S in vivo will become even more urgent. Moreover, there will undoubtedly be new avenues for therapeutic exploitation of the powerful anti-inflammatory, chemopreventative cytoprotective effects of H$_2$S [5,19].

References


