Forum Review Article

Anti-Inflammatory and Cytoprotective Actions of Hydrogen Sulfide: Translation to Therapeutics

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ABBREVIATIONS

ATP, adenosine triphosphate; BCA, β-cyanolalanine; CBS, cystathionine β-synthase; CHH, O-(carboxymethyl) hydroxalamine hemihydrochloride; COX, cyclooxygenase; CSE, cystathionine ϒ-lyase; GI, gastrointestinal; iNOS, inducible nitric oxide synthase; IL, interleukin; NaHS, sodium hydrosulfide; NF-kB, nuclear transcription factor-kB; Nrf2, nuclear factor (erythroid-derived 2)-like 2; NSAID, nonsteroidal anti-inflammatory drug; PAG, propargylglycine; TBZ, 4-hydroxythiobenzamide; TNF, tumour necrosis factor.

Abstract

Significance: There is a rapidly expanding body of evidence for important roles of hydrogen sulfide in protecting against tissue injury, reducing inflammation and promoting repair. There is also growing evidence that H_2S can be successfully exploited in drug development. Recent Advances: H_2S synthesis and degradation are regulated in circumstances of inflammation and injury so as to promote repair and re-establish homeostasis. Novel H_2S-releasing drugs exhibit enhanced anti-inflammatory and pro-restorative effects, while having reduced adverse effects in many tissues. Critical Issues: H_2S is a pleiotropic mediator, having effects on many elements in the inflammatory cascade and promoting the resolution of inflammation and injury. It also contributes significantly to mucosal defence in the gastrointestinal tract, and in host defence against infection. There is strong evidence that novel, H_2S-based therapeutics are safe and effective in animal models, and several are progressing through human trials. Future Directions: Better understanding the physiological and pathophysiological roles of H_2S continue to be restrained by the lack of simple, reliable methods for measurement of H_2S synthesis, and the paucity of highly selective inhibitors of enzymes that participate in endogenous H_2S synthesis. On the other hand, H_2S donors show promise as therapeutics for several important indications.

Introduction

For much of the past century, hydrogen sulfide was best known as an industrial pollutant and poison. As a result, H_2S has often been associated with and assumed to produce detrimental effects. However, as evidence has emerged for important physiological roles of H_2S in animals and plants, the notion that H_2S primarily exerts detrimental effects has been largely replaced by an understanding of the many beneficial effects produced by this mediator (42, 54, 55, 62). This re-appraisal has also been aided by more precise measurements of H_2S in mammals *in vivo*, which illustrated that previously reported levels were orders of magnitude overestimates (43). In the case of the role of H_2S in inflammation, much of the early literature focused on detrimental effects of high concentrations of H_2S. In the last decade, however, an important role of H_2S as an endogenous anti-inflammatory mediator has become increasingly clear. Moreover, several H_2S-releasing anti-inflammatory drugs are in development for treatment of a wide range of disease, and these drugs have shown considerable promise in relevant animal models (16, 19, 107). H_2S also plays a central role as a mediator of gastrointestinal (GI) mucosal defence and in promoting rapid repair of mucosal injury (105).

Hydrogen sulfide in inflammation

The cardinal signs of inflammation are redness, swelling, heat, pain and loss of function. One of the important contributions H_2S makes to inflammation is vasodilation (116), which accounts for the redness, and also contributes to swelling

(edema formation). Inhibitory effects of H_2S on phosphodiesterases may contribute to elevations in blood flow (15). While there are some reports of edema formation after injection of an H_2S donor directly into a tissue (10), most studies provide evidence for a reduction of vascular permeability and edema formation by H_2S (92, 115), particularly in circumstances of pre-existing inflammation. In a rat model of carrageenan-induced hind paw edema, for example, administration of NaHS, a H_2S donor, resulted in significant dose-dependent reductions of paw volume, while inhibition of H_2S synthesis significantly augmented the swelling response (115). Similarly, an H_2S-releasing derivative of diclofenac reduced carrageenan-induced paw edema in rats significantly greater than the effect observed with diclofenac itself (92).

The reduction of edema formation by H_2S donors may in part be due to the potent inhibitory effects of this mediator on leukocyte adherence to the vascular endothelium, and on transmigration of leukocytes across the endothelium (115). Zanardo *et al.* first demonstrated this action of H_2S using various H_2S donors (NaHS, Na_2S or Lawesson's reagent) and studying leukocyte-endothelial interactions by intravital microscopy in the mesenteric microcirculation of rats (115). They also demonstrated that suppression of endogenous H_2S synthesis led to a prompt increase in leukocyte adherence to the vascular endothelium (115). These results are consistent with observations in transgenic mice that produce lower-than-normal levels of H_2S. Thus, mice heterozygous for the cystathione β-synthase (CBS) gene display increased vascular permeability, reduced leukocyte rolling velocity and increased leukocyte adherence to the endothelium (51).

The H_2S-induced reduction of leukocyte adherence to the vascular endothelium may be attributable to direct effects on expression of intercellular adhesion molecule (ICAM)-1 and lymphocyte function-associated antigen (LFA) (37). H_2S has also been shown to suppress endothelial ICAM-1 expression in response to high blood-glucose concentrations (45)

Inhibitory effects of H_2S on leukocyte adherence to the vascular endothelium may be in part mediated via annexin-1, an anti-inflammatory and pro-resolution mediator (81, 85, 91). Exposure of human neutrophils to micromolar concentrations of an H_2S donor (NaHS) resulted in a robust translocation of annexin-1 from the cytosol to the membrane (14). This was accompanied by suppression of interleukin (IL)-1-induced leukocyte adhesion and emigration in mesenteric venules. However, when the same studies were performed in annexin-1-deficient mice, this inhibitory effect of H_2S was not apparent. Annexin-1 also appears to play a role in regulating endogenous AnxA1 on H_2S synthesis, as annexin-1-deficient mice were found to have a marked up-regulation of CBS and cystathionine γ-lyase (CSE) in a variety of tissues. A role of annexin-1 in mediating anti-inflammatory effects of H_2S in macrophages has also been demonstrated. Down-regulation of endotoxin-induced expression of iNOS and COX-2 by H_2S was absent in annexin-1-deficient mice (14).

Consistent with the inhibitory effects of H_2S on leukocyte adherence to the endothelium, infiltration of leukocytes to sites of injury/inflammation is also reduced by H_2S. This was demonstrated using a rat airpouch model in which carrageenan was used as the trigger for recruitment of leukocytes (107). Various

H_2S donors, at micromolar concentrations, reduced leukocyte infiltration significantly, while inhibitors of endogenous H_2S synthesis enhanced leukocyte infiltration. In a rat model of colitis, treatment with an H_2S-releasing derivative of mesalamine completely suppressed granulocyte infiltration, an effect that was not observed with mesalamine alone (39), but could be replicated with other H_2S donors (47, 71, 101)

Hydrogen sulfide can also exert inhibitory effects on other aspects of neutrophil behaviour. For example, it has been shown to scavenge hypochlorous acid, which can contribute to tissue injury at sites of inflammation (112). Palinkas et al. (78) recently reported that micromolar concentrations of H_2S could inhibit neutrophil myeloperoxidase activity. Myeloperoxidase is an enzyme that plays important roles in host defence against microbes, but is also believed to contribute significantly to tissue injury in the setting of inflammation (13). Thus, an inhibitory effect of H_2S on myeloperoxidase activity could result in diminished inflammation-associated tissue injury. However, it is also the possibility that this effect could increase susceptibility to bacterial invasion. Indeed, it is possible that sulfur-reducing bacteria may use H_2S in this manner as a defensive response against host myeloperoxidase activity. H_2S can also induce apoptosis of neutrophils (69), which is a critical step in the resolution of inflammatory responses (85).

Some anti-inflammatory actions of macrophages are also be enhanced by H_2S. Phagocytosis of *E. coli* was significantly increased in macrophages pre-treated with NaHS, and this donor also significantly enhanced the mobilization of macrophages towards a chemotaxin in a concentration-dependent manner (31).

H_2S donors have also been shown to down-regulate inflammatory mediator (IL-1β, IL-6, TNF-α, nitric oxide, etc.) production from macrophages (113).

The ability of H_2S donors to modulate expression of genes for many pro-inflammatory cytokines, chemokines and enzymes has largely been linked to effects on NF-κB activity. For example, there is considerable evidence of H_2S reducing COX-2 expression in settings of established inflammation, likely due in part to inhibition of NF-κB activity (36, 60). In the gastrointestinal tract, where there is a 'basal' level of low-grade inflammation related to the large numbers of microbes in the lumen, H_2S plays a role in maintaining constitutive COX-2 expression and prostaglandin synthesis. This is consistent with the roles of H_2S in maintaining mucosal integrity and in resolution of inflammation, since COX-2-derived eicosanoids are crucial mediators of these processes (85, 94) (Figure 1). Thus, treatment of healthy rats with inhibitors of H_2S synthesis was shown to result in a significant decrease in expression of COX-2, a decrease in mucosal synthesis PGE_2 synthesis, and an increase in mucosal inflammation (101).

Inhibition of NF-κB activity by H_2S can also lead to significant down-regulation of the production of a number of pro-inflammatory cytokines, chemokines, and enzymes that contribute to inflammation and tissue injury. For example, the slow-releasing H_2S donor, GYY4137, inhibited the secretion of TNF-α and IL-1β in an endotoxic shock model (63, 113). In rodent models of colitis, treatment with H_2S-releasing drugs significantly reduced tissue expression of TNF-α, IL-1β, interferon-ϒ, IL-2, IL-12 p40, RANTES and inducible nitric oxide synthase (iNOS), but did not affect expression of IL-10 (regarded as an anti-inflammatory

cytokine) (39, 101). In a study of biopsies from patients with ulcerative colitis, allyl disulfide significantly inhibited NF-κB activation and production of TNF-α (5), consistent with the above-mentioned animal studies.

Anti-nociceptive effects of hydrogen sulfide

One of the more controversial areas of research on H_2S as it pertains to inflammation is the role of this mediator in nociception. Several studies have demonstrated significant anti-nociceptive effects of H_2S donors in models of visceral pain (28, 29), but others have shown diametrically opposite effects (72). The reasons for the discrepancies are not clear, but may be to some extent related to the use of different concentrations of H_2S, different routes of administration, different methods for measuring pain, and in some cases, basing the results on the use of inhibitors of H_2S that lack selectivity (this issue is discussed in more detail below). It is noteworthy that one H_2S-releasing drug (GIC-1001) is nearing completion of a phase 2 human trial as a colonic analgesic (23). Significant anti-nociceptive effects of H_2S-releasing agents have also been demonstrated in models of peripheral pain (24, 32, 33).

We have examined the effects of H_2S on visceral pain using a well characterized, validated and objective cardio-autonomic endpoint (52, 97, 108). Gastric (or colonic) distention in rats leads to a significant decrease in heart rate, with the magnitude of the bradycardia correlating well with the degree of distention. Figure 2 summarizes results utilizing a well-characterized model of visceral pain in which the responses to gastric distention are quantified by changes

in heart rate. Distention led to a significant decrease in heart rate, which could be significantly attenuated by intraperitoneal administration of H_2S donors (NaHS or Lawesson's reagent) (Fig 2A and 2B). The H_2S donors did not affect gastric compliance (effect of NaHS shown in Fig 2C). A role of endogenous H_2S in modulating visceral nociception is demonstrated by the ability of L-cysteine to produce an anti-nociceptive effect in the model, and a reversal of that effect by co-administration of an inhibitor of CSE activity (Fig 2D).

Pro- versus Anti-Inflammatory Effects of H_2S: Why the Discrepancy?

There has been some controversy as to whether H_2S is an anti-inflammatory or a pro-inflammatory mediator. However, this is not a situation unique to H_2S, as similar debates have arisen in the literature with respect to a number of other pleiotropic inflammatory mediators, including prostaglandins and nitric oxide. Whether a mediator, such as H_2S, produces an effect that is viewed as anti-inflammatory or pro-inflammatory is to a large extent dependent on the context. Vasodilation induced by H_2S could be viewed as a beneficial effect (increasing resistance to tissue injury and promoting repair) or a detrimental effect (increasing edema formation). One must also consider the models that are used: effects of H_2S *in vitro* are, in general, less likely to be clinically relevant than effects of H_2S *in vivo*. On the other hand, with the *in vivo* models, there is a greater potential for H_2S to affect multiple targets, with the 'read-out' being a sum of a number of different, possibly opposing effects. Effects of H_2S in physiological settings may also differ markedly from effects in pathophysiological settings. For example, administration

of an inhibitor of H_2S synthesis may cause a negligible effect on systemic blood pressure in a healthy animal, but could rescue an animal from fatality in a model of endotoxic shock. The conclusion from the latter might be that H_2S is "bad", but one must consider the context and the relevance of the model to the human condition it attempts to reflect, the multiple targets that might be affected by H_2S.

Other factors that can contribute to apparent inconsistencies in the literature include differences in the doses of H_2S donors used, and the selectivity of inhibitors of H_2S synthesis. To illustrate the latter point, we examined the first 20 publications listed on PubMed in a search for the key words "PAG" and "hydrogen sulfide". PAG is the commonly used abbreviation for propargylglycine, which is widely used as a selective inhibitor of CSE. However, it is the L-isomer of PAG that inhibits CSE, while the R-isomer does not (48). Moreover, significant toxicities (particularly in the kidney) have been reported for *in vivo* use of the R-isomer of PAG (58, 89). Despite the wide commercial availability of L-PAG, we found that 17 of the 20 papers we examined specified that the racemate, D/L-PAG, was used, while the remaining 3 papers only referred to the use of "PAG". Thus, in at least 85% of cases, an inhibitor was used that is unquestionably non-specific, when a much more specific option (L-PAG) was available. In 17 of the 20 papers, no attempt was made to confirm that the effect of PAG was attributable to suppression of CSE activity, such as through the use of a second inhibitor of CSE, CSE-deficient mice, etc. In the *in vitro* studies, where stated, the concentrations of D/L-PAG used were between 0.5 and 1 mM, more than 10-times the IC_{50} for inhibiting CSE (3).

Similar problems can arise with inhibitors of CBS. For example, O-

(carboxymethyl)hydroxylamine hemihydrochloride (CHH) is widely used as a selective inhibitor of CBS, including by ourselves (70, 101). However, CHH can also inhibit aminotransferases (22), including cysteine aminotransferase, and can therefore inhibit synthesis of H_2S via the cysteine aminotransferase/3-mercaptopyruvate sulfurtransferase pathway (40).

H_2S in Cytoprotection

The ability of H_2S to protect cells from injury under various circumstances appears to be universal across the three domains of life. Plants utilize H_2S in defence against infection and extremes of climate (abiotic stress tolerance) and for promotion of growth and germination (9, 30, 87), while microbes (including archaebacteria) and animals use H_2S as a source of energy and in resisting and responding to damage. The ability of mitochondria to utilize H_2S as a 'metabolic fuel' (44) such that adenosine triphosphate (ATP) generation is continued in situations of hypoxia, is an ancient capacity that now is recognized as an important defensive response in many tissues, including the central nervous system (54), heart (34), liver (84) and kidney (4). Epithelial cells lining the gastrointestinal tract are reported to be the best adapted cells in the body for utilizing H_2S as an energy source (74), and it is possible that bacteria-derived H_2S may be used by these cells, both in health and disease (96). H_2S is produced throughout the GI tract (70), but there is evidence that microbe-derived H_2S contributes significantly to that measured in many tissues (41, 86).

Ulcer Healing

The detrimental effect of NSAIDs on healing of ulcers is an important clinical conundrum for which H_2S may provide a solution. When a patient taking NSAIDs develops an ulcer in the stomach or duodenum, they are typically advised to cease use of the NSAIDs. Of course, this results in a loss of the beneficial effects of the NSAIDs for the patient (reduction of pain and swelling). COX-2-derived PGs contribute importantly to ulcer healing (66, 75). Thus, use of NSAIDs significantly impairs the healing process, and this can lead to ulcer perforation and death. In studies using a mouse gastric ulcer model, celecoxib and naproxen significantly inhibited ulcer healing (93), mimicking the clinical scenario. However, administration of an H_2S-releasing derivative of naproxen significantly enhanced ulcer healing (93).

The ability of H_2S to promote repair of tissue injury likely involves several mechanisms, including promotion of angiogenesis (79). In the GI tract, there is rapid up-regulation of H_2S-producing enzymes, specifically at the sites of ulceration, and it has been clearly shown to promote healing and resolution of inflammation (40, 95, 101). Administration of H_2S donors can further accelerate ulcer healing, while inhibitors of H_2S have the opposite effect (95). Indeed, in an animal model of colitis, administration of inhibitor of H_2S synthesis over a period of several days led to death as a result of perforation of the colonic ulcers, and subsequent peritonitis (101). The site-specificity of the up-regulation of H_2S synthesis is intriguing. As illustrated in figure 3, up-regulation was not seen in immediately adjacent, inflamed tissues. Moreover, specifically at the sites of ulceration, there was a marked

decrease in rates of inactivation of H_2S (Figure 3). Along with the elevated H_2S synthesis, this would contribute to higher concentrations of H_2S at the sites where repair is needed. The decreased rates of inactivation of H_2S at these sites is not entirely surprising, given that oxidation of H_2S occurs primarily via mitochondrial sulfide quinone reductase, and this enzyme is most strongly expressed in intestinal epithelial cells (64, 74), which are destroyed at sites of ulceration. It is not yet clear which cells are responsible for the elevated expression of enzymes involved in synthesis of H_2S.

The ability of H_2S to increase blood flow to sites of injury likely also contributes to enhanced healing, and this may be in part mediated via induction of enzymes such as heme oxygenase-1 (25) and hypoxia-inducible factor-1 (47), as well as through stimulation of blood vessel growth (angiogenesis) via vascular endothelial growth factor (79).

GI Cytoprotection with H_2S

Our studies have mainly focused on the role of H_2S in cytoprotection of the mucosal lining of the GI tract. The question "why doesn't the stomach digest itself?" remains incompletely answered (26, 102), but it is now clear that H_2S contributes to many functions in these tissues (38) and is one of the most important mediators of mucosal defence against the relentless exposure to potentially erosive substances such as acid, digestive enzymes, bile, alcohol and various drugs. NSAIDs, which suppress mucosal synthesis of prostaglandins, are a major cause of gastrointestinal ulceration (103). A role for H_2S in mucosal defence is clear from studies

demonstrating that suppression of H_2S synthesis renders the mucosa much more susceptible to injury induced by a variety of factors, including NSAIDs (88), stress (1) and ischemia-reperfusion (65, 69). A new class of H_2S-releasing NSAID derivatives has been shown to produce negligible damage in the GI tract (Figure 4), even at doses many times greater than those required for anti-inflammatory effects (93), in circumstances in which mucosal defence is significantly impaired (93), and in circumstances in which co-administration of NSAIDs with other drugs produces substantially greater levels of injury (12). These novel drugs have been created by covalently linking an NSAID (e.g., ibuprofen, naproxen, diclofenac, ketoprofen, indomethacin, lumiracoxib) to an H_2S-releasing moiety (16, 102). Interestingly, administration of the two components (separately) rather than the intact compound does not result in protection against GI damage (87). A possible explanation for this observation is that the amount of H_2S released from the H_2S-releasing moiety is considerably greater when it is covalently linked to the NSAID than that released from the moiety alone (Figure 5). This effect was observed when the drugs were incubated in buffer, but to a much greater extent when the drugs were incubated in a liver homogenate (Figure 5). We have also observed increased release of H_2S from these compounds when incubated in the presence of reducing agents, such as dithiothreitol, glutathione and L-cysteine (unpublished).

The safety of the H_2S-releasing NSAIDs is observed throughout the GI tract, which is not the case for other approaches to GI protection against NSAID-induced injury. For example, co-administration agents that suppress gastric acid secretion, such as proton pump inhibitors and histamine H2 receptor antagonists, only reduce

NSAID-induced ulceration in the stomach and duodenum. Indeed, these drugs have recently been shown to markedly exacerbate NSAID-induced damage to the more distal small intestine (12, 101), where the damage is much more difficult to detect and to treat (106). Low-dose aspirin also exacerbates the GI damage caused by NSAIDs, and its use is common as a strategy to reduce the cardiovascular adverse events that can be triggered by conventional and COX-2-selective NSAIDs. In contrast, H_2S-releasing NSAIDs do no elicit small intestinal damage or bleeding when combined with low-dose aspirin (± proton pump inhibitors) (103, 106).

What is the mechanism of action of H_2S in protecting the GI mucosa? There are likely multiple mechanisms, as summarized in Table 1. A high level of mucosal blood flow is important to mucosal integrity, as it provides a mechanism for immediate buffering of acid that back-diffuses into the mucosa, thereby minimizing tissue damage, and facilitating rapid repair of any epithelial damage (99). Thus, the vasodilatory effects of H_2S likely contribute significantly to mucosal defense (37, 68). Inhibition of H_2S synthesis results in diminished gastric blood flow, and increases the susceptibility of the stomach to injury induced by NSAIDs (93). H_2S donors can prevent the decrease in gastric mucosal blood flow caused by NSAIDs, and thereby reduce the severity of NSAID-induced tissue injury (37, 96). While nitric oxide has been implicated in mediating some of the effects of H_2S (57), the protective effects of H_2S in the stomach are still evident when nitric oxide synthesis is inhibited (93). The gastro-protective effects of H_2S also do not appear to be mediated via ATP-sensitive potassium channels or TRPV1 channels, since the H_2S-

releasing NSAIDs continue to be gastric-sparing in rats pretreated with capsaicin to deplete sensory afferent neurons (93).

The inhibitory effects of H_2S on NSAID-induced leukocyte adherence to the vascular endothelium (37, 115) also contributes to prevention of injury by these agents, since leukocyte adherence is a very early and critical event in the pathogenesis of NSAID-induced GI injury (73, 98). This effect of H_2S appears to be due to a down-regulation of expression of ICAM-1 on endothelial cells and LFA-1 on leukocytes (37). The ability of H_2S to inhibit neutrophil myeloperoxidase-mediated tissue injury (78), to scavenge oxygen-derived free radicals (111) and to inhibit release of TNFα, all of which have been implicated in the pathogenesis of NSAID-induced enteropathy (8, 82, 83, 110), may also contribute to its cytoprotective effects.

There are several non-vascular effects of H_2S that contribute to mucosal defence. For example, gastroduodenal bicarbonate secretion, which helps to protect the stomach and upper small intestine from the damaging effects of gastric acid, has been shown to be H_2S-dependent (49). H_2S-dependent mitochondrial generation of ATP likely contributes to mucosal protection in settings of hypoxia in the GI tract (44, 55), including the protective effects of H_2S in the stomach during ischemia-reperfusion (65, 69) or cold-restraint stress (1). The insidious damage that NSAIDs can trigger in the small intestine has been suggested to be due to the uncoupling of mitochondrial respiration (67). The ability of H_2S to rescue mitochondrial function may therefore by a key mechanism underlying its protective effects against NSAID-induced enteropathy (103, 104). NSAID-induced enteropathy has also been shown

to be dependent upon enterohepatic circulation of the NSAID, and on the presence of bacteria in the intestine (103, 104). The relationship between enteric bacteria and enterohepatic recirculation of NSAIDs is complicated, as bacterial enzymes contribute significantly to the re-absorption of NSAIDs in the terminal ileum as well as to metabolism of bile acids. There is emerging evidence that H_2S can significantly influence these processes. Thus, H_2S donors can reduce the cytotoxicity of bile (on intestinal epithelial cells) and can alter the enteric flora (10, 76), effects that may account, at least in part, for the cytoprotective actions of H_2S in the small intestine.

Motta et al. (76) recently demonstrated that administration of an H_2S donor (diallyl disulfide) to rats led to significant increases intestinal mucus synthesis and epithelial cell expression of cathelicidin, an antimicrobial peptide critical for defence against bacterial infection (77). They also observed significant inhibitory effects of various H_2S donors on growth of several strains of intestinal bacteria (76).

As mentioned above, suppression of H_2S synthesis leads to an impairment of GI mucosal defence, in parallel with reduced COX-2 expression, reduced prostaglandin synthesis, enhanced pro-inflammatory cytokine production and elevated levels of granulocytes within the mucosa. Further evidence of the importance of H_2S in mucosal defence comes from studies utilizing iodoacetamide to induce inflammation in the stomach or colon (6, 84, 105). L-cysteine, the precursor for H_2S synthesis, is rapidly and avidly bound by iodoacetamide, thereby making it unavailable for conversion to H_2S (105). This results in significant granulocyte infiltration into the mucosa, ensuing mucosal injury (84).

H_2S has also been shown to activate the stress response pathway, up-regulating an array of detoxifying proteins and anti-oxidant enzymes (80, 114). The underlying mechanism for this effect may be the sulfhydration by H_2S of a protein (Keap1) that tonically suppresses Nrf2 activity (80, 114). These effects are consistent with an important role of H_2S in preserving mitochondrial integrity and function, particularly in circumstances of hypoxia or anoxia (17, 34, 55, 59). The protective effects of H_2S against ischemia/reperfusion-induced damage to gastric epithelial cells was recently shown to be mediated by suppression of Keap-1, via sulfhydration (46).

Therapeutic Applications of H_2S-Releasing NSAIDs

The most obvious indications for H_2S-releasing NSAIDs are those for which NSAIDs are currently used, and particularly chronic conditions where repeated use of NSAIDs is associated with significant adverse effects, such as gastrointestinal ulceration and bleeding (103, 107). The most common indications for NSAID use are osteoarthritis, rheumatoid arthritis, ankylosing spondylitis, post-surgical pain, gout, sports injuries, dental pain, dysmenorrhea and headaches. NSAIDs are widely used in veterinary practice for many of the same indications as in humans. Pre-clinical results suggest that the efficacy of H_2S-releasing NSAIDs is at least as good as existing NSAIDs (including selective COX-2 inhibitors), but with substantially reduced toxicity in the GI tract and other organs (12, 32, 33, 92, 93, 103).

Of course, the anti-inflammatory and cytoprotective actions of H_2S overlap, given that many damage processes include an inflammatory component;

inflammation being a defensive response to injury/infection that, when not properly resolved, will contribute to tissue damage (85). An example would be the neural injury that develops in the hours and days following spinal cord trauma, where the secondary inflammatory response accounts for considerable neural injury. In a mouse model of spinal cord injury, treatment with an H_2S-releasing anti-inflammatory drug (ATB-346) was found to result in a marked improvement of motor function, which correlated well with the enhanced anti-inflammatory effects of the drug during the 24 hours after trauma (18) (Figure 6a). The effects of the H_2S-releasing drug were markedly superior to the parent anti-inflammatory drug, underscoring the profound beneficial effects of delivery of small amounts of H_2S to the injured tissue. Indeed, the H_2S-releasing drug was significantly more effective than the parent drug (naproxen) in terms of reduction of histological damage, number of activated microglia, granulocyte infiltration (Fig 6b), and expression of TNFα, IL-1β, COX-2 and iNOS (18). Treatment with the H_2S-releasing moiety alone did not produce significant beneficial effects in this model (18), suggesting that the combination of H_2S release and COX inhibition was necessary to achieve the desired effects.

Chronic use of NSAIDs has long been known to be associated with significantly lower rates of various types of cancer, most notably colorectal cancer (2, 7, 27, 90). This effect is presumably related to the anti-inflammatory actions of the NSAIDs, and possibly specifically related to suppression of COX-2 activity (109). Unfortunately, the ability of this class of drugs to induce ulceration and bleeding in the GI tract greatly limits their use for chemoprevention. This may be another

circumstance in which the GI-sparing properties of H_2S-releasing NSAIDs could be exploited. However, several studies in recent years have provided evidence that H_2S-releasing NSAIDs, as well as being GI-safe, have enhanced chemopreventative effects in models of colorectal cancer and other types of cancer (20, 21, 61). Figure 7 illustrates the significantly improved chemopreventative activity of a H_2S-releasing derivative of naproxen in a mouse model in which pre-cancerous lesions were induced via administration of a carcinogen (azoxymethane) (33). This compound (ATB-346) dose-dependently reduced the number of aberrant crypt foci in the colon. It was significantly more effective than the parent NSAID (naproxen) at all doses tested. As in the case of spinal cord trauma, administration of the H_2S-releasing moiety of ATB-346 alone did not confer any beneficial effects in the colon cancer model, once again suggesting the need for the actions of both the NSAID and H_2S-releasing components of the drug (Figure 7).

NSAIDs are not the only class of drugs that can be improved through coupling to an H_2S donor. Improved activity of an H_2S-releasing derivative of mesalamine in rodent models of colitis (39, 101). In this case, the parent drug is quite safe, and very high doses are often employed in a clinical setting without significant adverse effects. However, mesalamine is not very potent (hence the use of high doses). H_2S-releasing derivatives of mesalamine exhibited more potent and broader anti-inflammatory effects than the parent drug, and in models of colitis they markedly accelerate resolution of tissue injury and inflammation. Also, as mentioned above, an H_2S-releasing compound (GIC-1001) exhibiting enhanced anti-nociceptive effects in visceral pain models is now in phase 2 clinical trials as a colonic analgesic (25).

Conclusions

Research over the past decade has further demonstrated the fundamental roles of H_2S in tissue defence and responses to injury (42, 54, 55, 62). Inflammation is a crucial defensive response to injury. H_2S modulates most, if not all, components of an inflammatory response, and also has the capacity to lessen tissue injury (such as through its ability to rescue mitochondrial function) and accelerate repair. These important actions of H_2S make it an attractive drug to exploit in drug design.

The powerful protective effects of H_2S, such as those in the GI tract, can be beneficial in several therapeutic applications. The protective effects of H_2S are remarkable – in the case of small intestinal damage caused by NSAIDs, no other agent has been shown to so profoundly reduce the injury caused by these drugs. This type of injury is of considerable clinical significance given the widespread use of NSAIDs, mainly by patients with multiple risk factors for intestinal ulceration, including the use of other drugs that can exacerbate NSAID-induced enteropathy, such as low-dose aspirin and inhibitors of gastric acid secretion (100, 106). Increasing the safety of NSAIDs via coupling to an H_2S-releasing moiety offers the possibility of using these compounds as chemopreventative agents in several forms of cancer.

The restorative effects of H_2S are also remarkable, and have been shown to significantly accelerate the repair of tissue injury and to enhance orderly resolution

of inflammation. The value of novel H_2S-based compounds as therapeutics for humans will soon be known, as prototypes advance through clinical trials.

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FIGURE LEGENDS

Figure 1: Hydrogen sulfide (H_2S) production in the intestine is important for maintenance of mucosal integrity, in part via cyclooxygenase (COX)-2-dependent prostaglandin (PG)E_2 synthesis. Inhibition of H_2S synthesis, such as with β-cyanolalanine (BCA), results in significant reductions in COX-2 expression and PGE_2 synthesis, and an increase in mucosal inflammation (measured by tissue myeloperoxidase (MPO) activity). Data are shown as mean SEM with at least 5 rats per group (*$p<0.05$ versus vehicle-treated; ANOVA and Bonferroni test).

Figure 2: Anti-nociceptive effects of hydrogen sulfide (H_2S) in a rat model of visceral pain. Gastric distention results in a significant decrease in heart rate, which can be significantly reduced by intraperitoneal administration of H_2S donors such as NaHS (panel A; 60 μmol/kg) and Lawesson's reagent (Panel B; 30 μmol/kg). The H_2S donors did not cause a change in gastric compliance, which would obfuscate the pain measurements (panel C for NaHS). Endogenous H_2S can also reduce distention-induced visceral pain (panel D), as shown by the effect of L-cysteine (50 mg/kg), which was blocked by pretreatment with an inhibitor of cystathionine ϒ-lyase (L-propargylglycine; L-PAG; 50 mg/kg). Data are shown as mean ± SEM, with 5-6 rats per group (*$p<0.05$ versus all other groups; ANOVA and Bonferroni test).

Figure 3: Site-specific decrease in degradation of hydrogen sulfide (H_2S) in experimental colitis in rats. Panel A shows examples of colonic tissue from a healthy rat and a rat with hapten-induced colitis. The arrows illustrate the location

of sampling for healthy tissue (brown), ulcerated tissue (orange) and immediately adjacent non-ulcerated, but inflamed tissue (blue). The bottom panel shows the recovery of H_2S from samples of homogenized mucosal tissue from the three regions. As described in detail elsewhere (40), NaHS (33 mL of 30 mM NaHS) was added to the homogenized tissue samples 30 min later the concentration of H_2S in the homogenates wad determined. A greater recovery of H_2S would suggest reduced degradation/sequestration. Recovery of H_2S was significantly greater from ulcerated tissue than from healthy tissue (***$p<0.001$) and from the inflamed, but non-ulcerated tissue ($^{\alpha}p<0.05$). Each group consisted of at least 5 rats and the data are shown as mean ± SEM. ANOVA and Dunnett's Multiple Comparison Test were used to compare the groups of data.

Figure 4: Naproxen administration (orally) as a single dose caused extensive gastric damage, while repeated administration over several days resulted in severe ulceration in the small intestine. A hydrogen sulfide (H_2S)-releasing derivative of naproxen (ATB-346), at an equimolar dose, did not cause gastric or intestinal damage, despite producing comparable inhibition of cyclooxygenase activity. While not causing gastric of intestinal injury, pretreatment with ATB-346 immediately before naproxen did not confer any protective effect in the stomach or intestine. Results are presented as the mean ± SEM, with 6 mice per group.

Figure 5: Release of hydrogen sulfide (H_2S) *in vitro* from ATB-338, a derivative of diclofenac. ATB-338 consists of diclofenac linked to 4-hydroxythiobenzamide

(TBZ). Release of H_2S from ATB-338 was significantly greater than that from TBZ (*$p<0.05$). When incubated in a liver homogenate, the release of H_2S from TBZ and from ATB-338 were significantly greater than when the compounds were incubated in buffer ($^{\Psi}p<0.05$), and release of H_2S from ATB-338 was substantially greater than that from TBZ.

Figure 6: Accelerated recovery of motor function after spinal cord injury in mice through treatment with a hydrogen sulfide (H_2S)-releasing anti-inflammatory drug (ATB-346). Panel A: The motor function in mice following spinal cord injury was blindly scored using the Basso Mouse Scale. Daily treatment with equimolar (30 μmol/kg) doses of naproxen or ATB-346 resulted in significant (*$p<0.05$) improvement of the motor function score as compared to the vehicle-treated group, or the group treated only with the H_2S-releasing moiety of ATB-346 (TBZ). The improvement of motor function with ATB-346 treatment was significantly greater than that of naproxen ($^{\Psi}p<0.05$). Panel B: Spinal cord injury resulted, within 24 hours, in significant inflammation of the white matter at the site of trauma, with edema and extensive granulocyte infiltration, as indicated by the significant increase in myeloperoxidase activity (MPO) as compared to that in sham-treated mice (**$p<0.01$, ***$p<0.001$). Treatment with ATB-346 resulted in a marked reduction ($^{\alpha\alpha\alpha}p<0.001$) in MPO activity as compared to vehicle-treated rats, while treatment with naproxen or TBZ had no effect. This graph was constructed using previously

published data (18). Data are shown as mean ± SEM with 5-10 mice per group (ANOVA and Bonferroni test).

Figure 7: A hydrogen sulfide (H_2S)-releasing derivative of naproxen dose-dependently reduces the number of colonic aberrant crypt foci in the mouse, with greater effect than naproxen itself. Aberrant crypt foci, a pre-cancerous lesion in the colon, were induced through weekly administration of azoxymethane. The mice received daily oral treatment with vehicle, ATB-346, naproxen or the H_2S-releasing moiety of ATB-346 (4-hydroxythiobenzamide; TBZ). Naproxen was given at 10 mg/kg, and the other compounds at equimolar doses. The green boxes represent the mean number of aberrant crypt foci (% control) in mice treated with TBZ (no significant effect at any dose). Results are presented as the mean ± SEM, with at least 5 mice per group (*$p<0.05$ versus vehicle. $^{\Psi}p<0.05$ versus the corresponding dose of naproxen; ANOVA and Dunnett's Multiple Comparison Test).

TABLE 1
Mechanisms of GI-Protective Actions of Hydrogen Sulfide

Action	References
Inhibits leukocyte adherence/extravasation	115
Stimulates mucus and bicarbonate secretion	49, 76
Prevents mitochondrial damage	34, 55, 74
Enhances anti-microbial defence	76
Scavenges oxygen-derived free radicals	111
Activates anti-oxidant response elements	46
Inhibits neutrophil-mediated tissue injury (myeloperoxidase activity)	78
Metabolic fuel for intestinal epithelial cells	74
Reduces damage-associated TNF release	39
Reduces cytotoxicity of bile	12

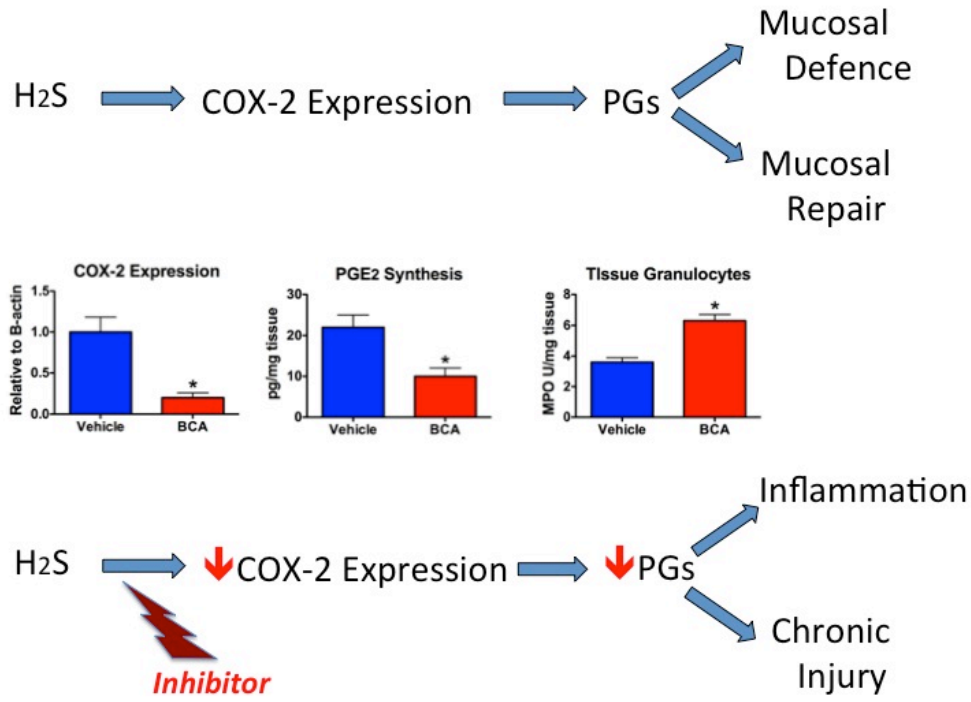
H2S
COX-2 Expression
PGs
Mucosal Defence
Mucosal Repair
COX-2 Expression
Relative to B-actin
1.5
1.0
0.5
0.0
*
Vehicle
BCA
PGE2 Synthesis
pg/mg tissue
30
20
10
0
*
Vehicle
BCA
TIssue Granulocytes
MPO U/mg tissue
8
6
4
2
0
*
Vehicle
BCA
H2S
COX-2 Expression
PGs
Inflammation
Chronic Injury
Inhibitor

Figure 1

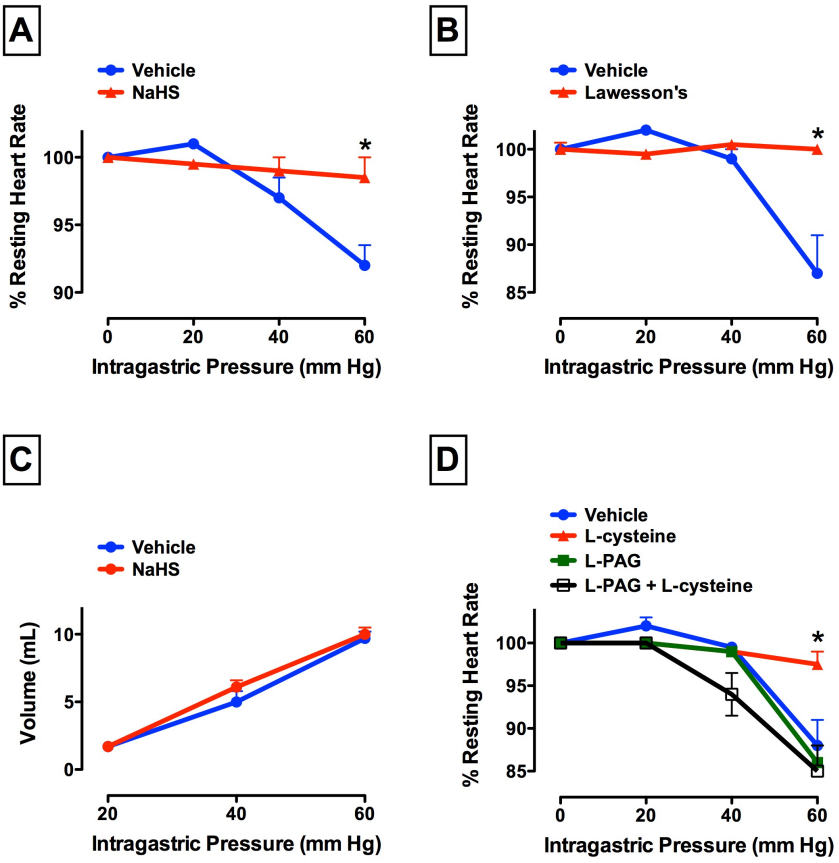
A
Vehicle
NaHS
% Resting Heart Rate
100
95
90
*
0
20
40
60
Intragastric Pressure (mm Hg)
B
Vehicle
Lawesson's
% Resting Heart Rate
100
95
90
85
*
0
20
40
60
Intragastric Pressure (mm Hg)
C
Vehicle
NaHS
Volume (mL)
10
5
0
20
40
60
Intragastric Pressure (mm Hg)
D
Vehicle
L-cysteine
L-PAG
L-PAG + L-cysteine
% Resting Heart Rate
100
95
90
85
*
0
20
40
60
Intragastric Pressure (mm Hg)

Figure 2

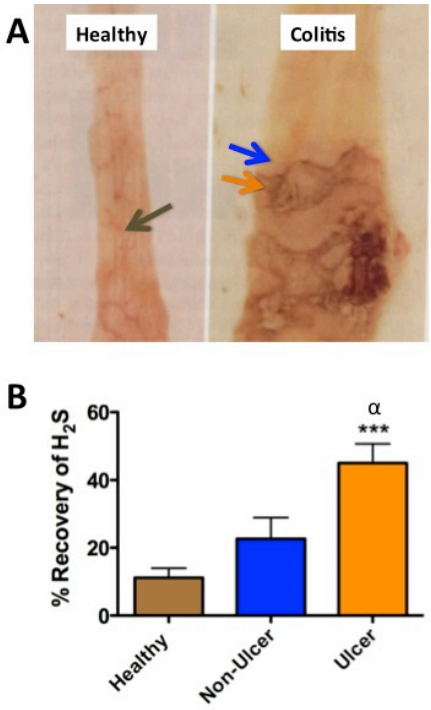
A
Healthy
Colitis
B
% Recovery of H_2S
60
40
20
0
α
***
Healthy
Non-Ulcer
Ulcer

Figure 3

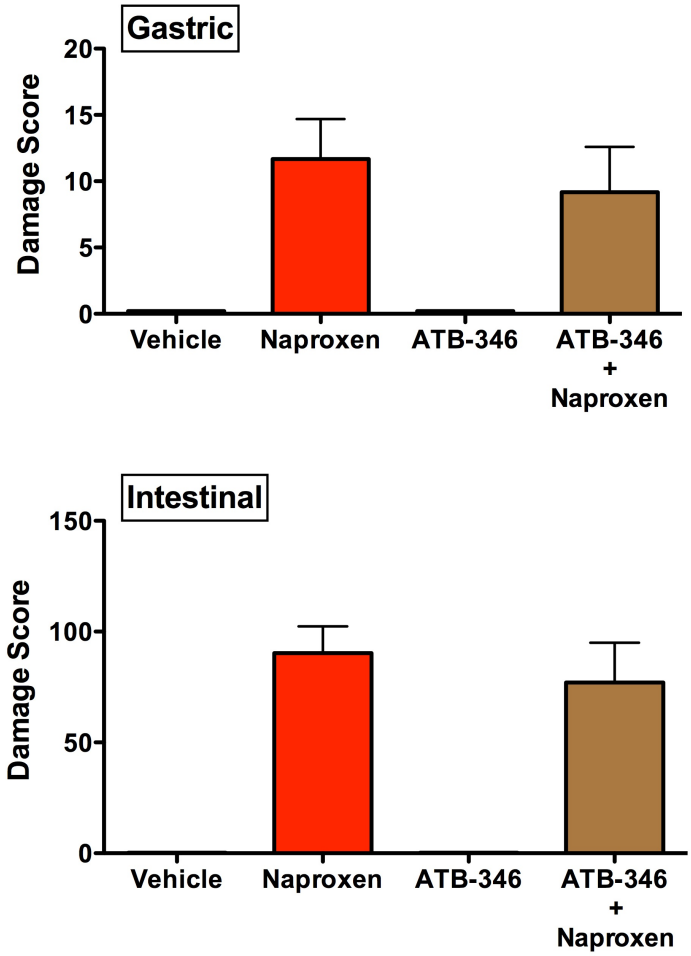
Gastric
Damage Score
20
15
10
5
0
Vehicle
Naproxen
ATB-346
ATB-346 + Naproxen
Intestinal
Damage Score
150
100
50
0
Vehicle
Naproxen
ATB-346
ATB-346 + Naproxen

Figure 4

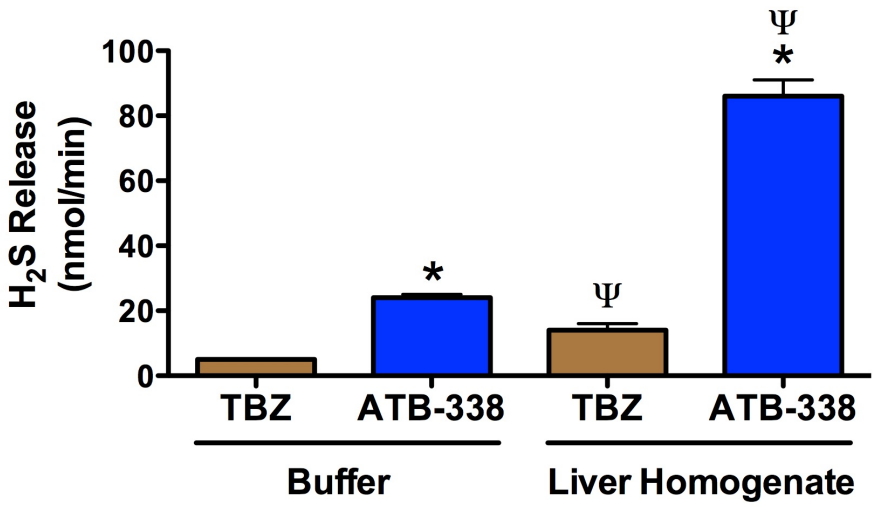
Ψ
*
100
80
60
40
20
0
H_2S Release
(nmol/min)
*
Ψ
TBZ
ATB-338
TBZ
ATB-338
Buffer
Liver Homogenate

Figure 5

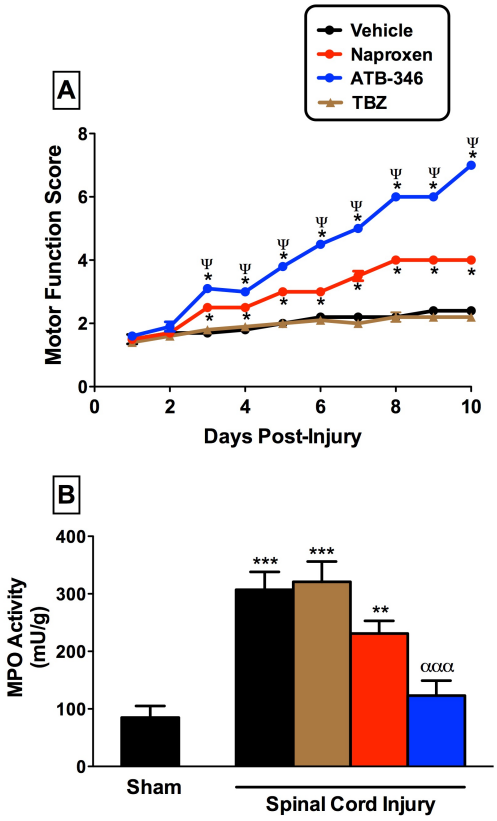
A
Vehicle
Naproxen
ATB-346
TBZ
Motor Function Score
Days Post-Injury
B
MPO Activity (mU/g)
Sham
Spinal Cord Injury

Figure 6

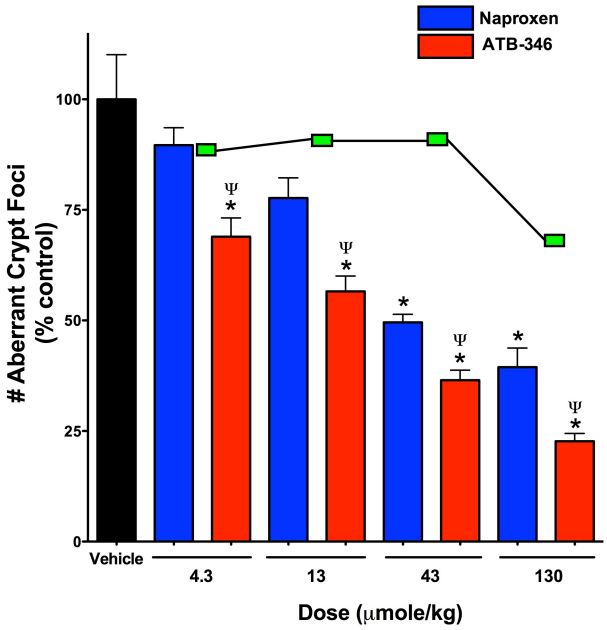
Naproxen
ATB-346
Aberrant Crypt Foci
(% control)
100
75
50
25
0
Vehicle
4.3
13
43
130
Dose (μmole/kg)
Ψ
*

Figure 7