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REVIEW ARTICLE

Nitric oxide in the gastrointestinal tract: opportunities for drug development

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Nitric oxide (NO) plays important roles in gastrointestinal mucosal defence, as well as in the pathogenesis of several gastrointestinal diseases (e.g. irritable bowel syndrome and inflammatory bowel disease). The potent cytoprotective effects of NO have been demonstrated in a range of animal models. However, in some disease states, inhibition of NO synthesis is beneficial. Several attempts have been made to develop drugs for ulcerative and/or inflammatory disorders of the gastrointestinal tract, with varying degrees of success. Covalently linking a NO-releasing group to non-steroidal anti-inflammatory drugs or to drugs used in the treatment of inflammatory bowel disease and irritable bowel syndrome has shown some benefit, although no drug of this type has yet been fully developed.

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Abbreviations

CRD, Colorectal Distention; GI, gastrointestinal; IBD, inflammatory bowel disease; IBS, irritable bowel syndrome; L-NAME, L-nitroarginine methyl ester; NEP, neutral endopeptidase; NSAID, nonsteroidal anti-inflammatory drug; SNP, single nucleotide polymorphism; Th, T helper; TNBS, trinitrobenzene sulfonic acid; TRPV, transient receptor potential cation channel subfamily V member; VEO, very early onset

As in most other tissues, **NO** plays key roles in regulating a wide range of functions in the gastrointestinal (GI) tract in both health and disease. These include contributions of NO to the maintenance of mucosal integrity, through modulation of numerous components of 'mucosal defence' and through regulation of secretion and smooth muscle function. The roles of NO in pathophysiological conditions are also substantial, particularly with respect to regulating mucosal inflammation, enteric pain and responses to injury. There are many poorly managed diseases of the GI tract, such as inflammatory bowel disease (IBD), for which NO-based therapies hold significant promise. In this review, the contributions of NO to mucosal defence and disease are reviewed, with examples provided of attempts to develop NO-based treatments for some prevalent GI disorders.

As in other tissues, NO can be produced by three **NOS** enzymes in the GI tract. **Neuronal NOS (nNOS** also known as NOS1) is the 'cytokine-inducible', neuron-associated form of the enzyme, primarily contributing to regulation of smooth muscle function and pain. **Inducible NOS (iNOS** also known as NOS2) is the 'inducible' form of the enzyme, primarily involved in responses to inflammation and injury. **Endothelial NOS (eNOS** also known as NOS3) is the 'endothelial' form of the enzyme, which plays particularly important roles in the GI tract in regulating mucosal defence from injury and vasodilation.

Cytoprotection and mucosal defence

The concept of GI cytoprotection was introduced by Andre Robert in 1968, referring to the ability of prostaglandins (PGs), in minute amounts, to greatly increase the resistance of the stomach to injury induced by a wide range of agents, including non-steroidal anti-inflammatory drugs (NSAIDs), **ethanol**, high concentrations of acid and bile salts (Robert *et al.*, 1968). In 1989, the first evidence that NO could profoundly increase GI mucosal resistance to injury was published (MacNaughton

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et al., 1989). Topical application of a 0.01% solution of NO significantly reduced the severity of mucosal damage induced by topical application of 70% ethanol. This protective effect was transient, consistent with the very short half-life of NO. A 5 min delay between the administration of the NO and the administration of the ethanol was sufficient for the protective effect to be lost. No loss of the protective effect was observed with pretreatment with **indomethacin**, precluding the involvement of endogenous PGs in the protective effects of the NO. NO donors such as **sodium nitroprusside** and **glyceryl trinitrate** were shown to elicit longer acting protection against gastric damage induced by oral administration of ethanol. However, intravenous infusion of 1% methylene blue significantly increased the susceptibility of the mucosa to damage induced by topical 20% ethanol (MacNaughton *et al.*, 1989).

Numerous subsequent studies confirmed these observations and established roles for NO in activating several elements of 'gastric mucosal defence', including mucus and bicarbonate secretion, reactive hyperaemia and the formation of a 'mucoid cap' over sites of epithelial damage that promotes rapid re-epithelialization after injury (Wallace, 2008). Mucus acts as a lubricant to reduce physical and chemical abrasion of the mucosa. It is secreted by GI epithelial cells and goblet cells. As well as reducing epithelial damage induced by acid and bile, it provides an important barrier to bacterial invasion of the mucosa. An increase in the thickness of the mucus layer is a normal defensive response to luminal insults. In addition, mucus traps secreted bicarbonate and plasma on the surface of the epithelium (Takeuchi *et al.*, 2011). Even in the very low pH environment of the stomach, this can provide a near-neutral pH micro-environment that is conducive to epithelial protection and repair. NO stimulates epithelial mucus secretion *via* activation of **guanylyl cyclase** (GC; Brown *et al.*, 1992), and **carbachol**-induced gastric mucus release is also mediated *via* NO (Price *et al.*, 1994).

The secretion of fluid by GI epithelial cells also dilutes any noxious substances in the lumen. Fluid secretion across the GI epithelium is mainly osmotically driven by the active transport of chloride ions into the lumen. This process is regulated by numerous soluble mediators and neurotransmitters, including NO (MacNaughton, 1993; Perdue and McKay, 1994). The effects of NO on GI secretion are not always stimulatory: low concentrations of NO are stimulatory while high concentrations are inhibitory. Indeed, NO also plays a key role in mediating the long-term impairment of epithelial secretion that can be observed after a bout of intestinal inflammation (Asfaha *et al.*, 1999, 2001).

Maintenance of perfusion of GI tissues with blood is crucial to tissue integrity, particularly in the stomach and duodenum, where back-diffusion of acid can result in extensive damage and bleeding. A rapid and well-characterized hyperaemic response is triggered by acid back-diffusion, which can prevent or limit mucosal injury. This response is mediated by sensory afferent nerves underlying the epithelium. The entry of acid into the lamina propria (Holzer and Sametz, 1986) triggers the release of calcitonin gene-related peptide (CGRP) from these neurons, resulting in the immediate dilation of submucosal arterioles. This facilitates both the dilution and buffering of the acid that has 'back-diffused' into the mucosa acid (Lippe and Holzer, 1992). NO mediates this vascular response to CGRP. Thus, administration of an inhibitor of NO synthesis abolished the reactive hyperaemic response, resulting in a marked increase in the susceptibility of the mucosa to damage (Lippe and Holzer, 1992).

The GI tract, particularly distal to the duodenum, is essentially in a state of chronic, low-grade inflammation. This is due to the ongoing interaction (and transepithelial migration) of luminal bacteria and their products with the mucosal immune system. Leukocytes can be stimulated to extravasate from mucosal blood vessels by chemotaxins that are released from bacteria, and this process can result in damage to the blood vessels and surrounding tissue and further generation of chemotaxins. NO plays a significant role in modulating leukocyte adherence to the vascular endothelium and in maintaining blood flow to the tissue (Kubes *et al.*, 1991). Inhibition of NO synthesis results in a marked increase in leukocyte adherence to the endothelium (Banick *et al.*, 1997), which can contribute significantly to mucosal injury (Wallace *et al.*, 1990). NO can inhibit expression of the ß-2 adhesion molecules on neutrophils (Davenpeck *et al.*, 1994) and P-selectin on the vascular endothelium (Kubes *et al.*, 1991). Adherence of leukocytes to the vascular endothelium in response to administration of a chemotactic factor can also be suppressed by administration of an NO donor (Wallace *et al.*, 1999).

As would be expected, considering the many beneficial effects of NO in GI mucosal defence, inhibition of NO synthesis has many detrimental effects. These include significant impairment of ulcer healing throughout the GI tract (Konturek *et al.*, 1993; Elliott *et al.*, 1995). Nevertheless, NO donors accelerate the healing of these lesions (Elliott *et al.*, 1995), through increased epithelial cell migration and proliferation and *via* enhancement of collagen deposition by fibroblasts (Schaffer *et al.*, 1996). Another mechanism through which NO can enhance healing of ulcers is through maintenance of blood flow at the margin of the wound. Ulcer healing requires the proliferation and differentiation of epithelial cells at the ulcer margin, which is dependent upon adequate blood flow. A reduction of blood flow in these circumstances can result in retardation of ulcer healing (Papapetropoulos *et al.*, 2015). Ulcer healing also requires the growth of new blood vessels in the ulcer margin (angiogenesis), and NO is a potent stimulant of that process (Papapetropoulos *et al.*, 2015).

Drug development: NO-releasing NSAIDs

The use of NSAIDs is associated with a high incidence of bleeding and ulceration in both the upper and lower GI tract. However, the underlying mechanisms for the mucosal injury differ significantly between these regions. In the stomach and proximal duodenum, NSAID-induced ulceration is largely acid-dependent and directly linked to the degree of suppression of **COXs** that is achieved with the NSAID (Wallace, 2012). Upper GI damage triggered by NSAIDs can usually be prevented by administration of proton pump inhibitors or histamine H_2 receptor antagonists (i.e. the damage is acid-dependent). Distal to the proximal duodenum, the ulceration and bleeding associated with NSAID use is not acid-dependent and is much less COX-dependent than that in the upper GI tract. NSAID-enteropathy is directly related to (i) enterohepatic circulation of NSAID-glucuronides that are formed after absorption of the NSAID, (ii) topical irritation of the epithelium by the NSAID and NSAID-glucuronides and (iii) invasion of the epithelium by enteric bacteria (Wallace, 2012).

The observations that NO and NO donors could protect the stomach from NSAID-induced damage (MacNaughton *et al.*, 1989) triggered research into the possibility that linking a NO-releasing moiety to an NSAID may result in a substantially less GI-toxic drug than the NSAID itself, as long as the conjoint drug retained the ability to suppress COX activity. A French company, NicOx S.A., developed and characterized the effects of a number of such drugs. Several were found to be markedly safer than conventional NSAIDs when tested in rat models (Wallace and Del Soldato, 2003), while retaining comparable anti-inflammatory effects to those seen with the 'parent' drugs. For example, NO-releasing derivatives of **diclofenac** and **naproxen** exhibited comparable COX suppression and anti-inflammatory effects as the parent drugs, with negligible GI damage (Wallace *et al.*, 1994; Elliott *et al.*, 1995; Davies *et al.*, 1997). One of the more significant adverse effects of NSAIDs in a clinical setting is the impairment of ulcer healing that can occur, leading to pronounced bleeding (since NSAIDs inhibit platelet aggregation) and a significant risk of perforation. As shown in Figure 1, studies in rats demonstrated that administration of an NSAID (diclofenac) to rats in which a gastric ulcer had been induced resulted in impairment of ulcer healing and significant bleeding (the latter being evident from a substantial decrease (~36%) in haematocrit over the course of the 1 week treatment). In contrast, administration of the NO-releasing derivative of diclofenac ('nitrofenac') actually accelerated ulcer healing despite suppressing PG synthesis and did not cause significant bleeding (i.e. no significant change in haematocrit) (Elliott *et al.*, 1995). A wide range of NO-releasing NSAIDs were synthesized and tested in models such as those described above, with comparable safety and efficacy profiles as seen with nitrofenac (Wallace and Del Soldato, 2003).

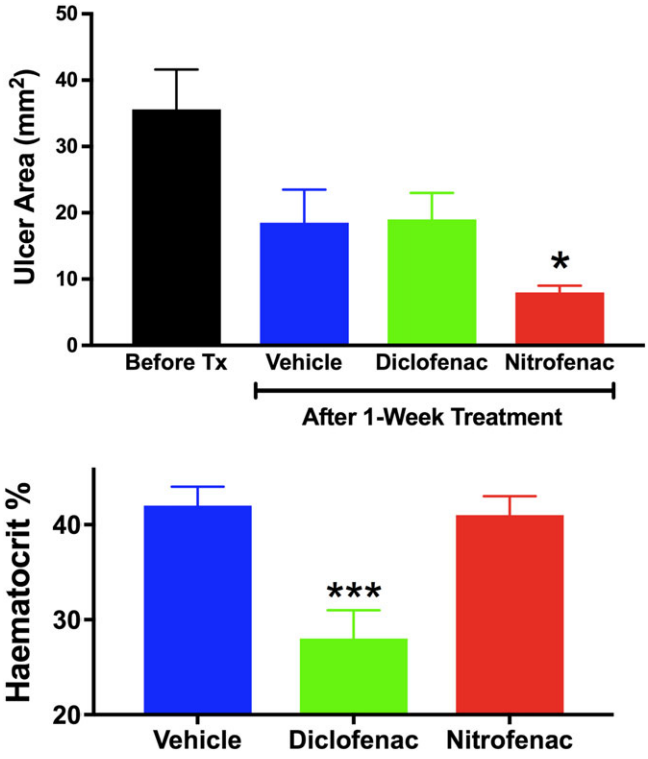

Figure 1

Upper panel: An NO-releasing derivative of diclofenac ('nitrofenac') significantly accelerated healing of experimentally-induced gastric ulcers in rats, as compared to the treatment with vehicle or with diclofenac itself. Equimolar doses of diclofenac and nitrofenac were administered once daily to rats over a period of 7 days after ulcer induction. Ten rats per group. *$P < 0.05$ versus the vehicle- and diclofenac-treated groups. Lower panel: Once daily administration of diclofenac over 7 days resulted in a significant (***$P < 0.001$) decrease in haematocrit, consistent with the haemorrhagic lesions observed in the GI tract, as compared to the vehicle- and nitrofenac-treated groups. Diclofenac and nitrofenac were administered at equimolar doses (5 and 7.5 mg·kg^{-1}, respectively; $n = 10$ per group). From Elliott *et al.* (1995).

An NO-releasing **naproxen** derivative ('**naproxcinod**') was eventually selected by NicOx for clinical development, in part because naproxen is one of the most commonly prescribed NSAIDs. In rat studies, naproxcinod had been shown to produce profoundly less injurious effects to the stomach than naproxen, not to significantly impair healing of preformed ulcers, and to spare the small intestine of damage when administered daily for 18 days (Davies *et al.*, 1997). Interestingly, naproxcinod exhibited superior anti-inflammatory and analgesic effects to equimolar doses of naproxen (Davies *et al.* 1997). The observed ability of NO-releasing NSAIDs to inhibit the expression of iNOS may have contributed to the enhanced analgesic effects of these drugs versus the 'parent' NSAID (Cirino *et al.*, 1996).

Early stage clinical trials of naproxcinod were similarly promising (Hawkey *et al.*, 2003). It elicited significantly fewer upper GI erosions than diclofenac (average of 4 vs. 12; $P < 0.0001$) (Hawkey *et al.*, 2003). Also, while naproxen administration caused a significant increase in small intestinal permeability (a marker of small intestinal injury), naproxcinod did not (Hawkey *et al.*, 2003). Naproxcinod progressed to phase 2 and 3 clinical trials, where comparable efficacy to naproxen was observed in patients with osteoarthritis (Lohmander *et al.*, 2005). However, the extent of reduction of upper GI damage as compared to conventional NSAIDs was not as profound as had been observed in animal studies. That observation, together with the emergence of selective **COX-2** inhibitors, led to the development of naproxcinod being halted.

NicOx continue to develop NO-releasing anti-inflammatory drugs, but with a focus on ocular, rather than GI, inflammation. VYZULTA™ (**latanoprostene bunod** ophthalmic solution) is a dual-acting NO-donating **$PGF_{2\alpha}$** analogue for reducing intraocular pressure, with significantly enhanced activity in lowering intraocular pressure as compared to latanaprostene.

Inflammatory bowel diseases

The role of NO in the treatment and pathogenesis of IBD is complex. Some studies in experimental models demonstrated beneficial effects of treatment with NO donors (Wallace *et al.*, 1999; Lund and Scholefield, 1997), while others showed beneficial effects of suppression of endogenous NO production (Hogaboam *et al.*, 1995; Aiko *et al.*, 1998). These divergent results drew attention to the potential

for NO derived from the different isoforms of NOS to behave very differently from one another. In particular, NO derived from iNOS appears to drive several detrimental effects in the context of GI inflammation. For example, the expression and activity of iNOS are markedly increased in colitis IBD and in experimental colitis (Soufli *et al.*, 2016) and appear to drive inflammation and the associated expression of pro-inflammatory cytokines (Soufli *et al.*, 2016). Nevertheless, anti-inflammatory and immunoregulatory cytokines are largely responsible for down-regulating the expression of iNOS, and a positive correlation has been observed in IBD patients between NO production and increased pro-inflammatory cytokine levels (**TNF-α**, **IL-6**, IL-17, **IL-12** and **IFN-γ**). In animal studies, NO has been shown to contribute to immune regulation by attenuating Th1 responses and inducing the expression of Th2-derived cytokines such as **IL-10** and **IL-4** (Wei *et al.*, 1995; Dimmeler and Zeiher, 1997; Coleman, 2001). Selective suppression of iNOS activity in animals has been shown to promote resolution of intestinal inflammation (Hogaboam *et al.*, 1995; Aiko *et al.*, 1998). Moreover, increased production of NO from iNOS was shown to be responsible for long-lasting impairment of intestinal secretory function, which may underlie post-inflammatory gut dysfunction (Asfaha *et al.*, 1999, 2001). In rat studies, the epithelium appeared histologically normal several weeks after a bout of colitis (induced chemically), but the ability of the epithelium to secrete elements in response to a range of agonists was significantly impaired (Asfaha *et al.*, 1999). Epithelial secretion (of fluid, mucus, etc.) is a significant component of mucosal defence, aimed at minimizing bacterial translocation. These rats had increased levels of colonic aerobic bacterial (16-fold), and the levels of bacterial translocation into the mucosa and lymph nodes were increased threefold (Asfaha *et al.*, 2001). The expressions of iNOS mRNA and iNOS activity were significantly increased, and treatment with a selective iNOS inhibitor normalized epithelial secretory responses (Asfaha *et al.*, 1999). The post-colitis bacterial translocation could be prevented by treatment with an inhibitor of iNOS activity (Asfaha *et al.*, 2001).

A clinical study of a rare form of IBD has further highlighted the importance of NO in the pathogenesis of this disease. Dhillon *et al.* (2014) suggested that iNOS may be particularly important in a specific subset of IBD patients who manifest particularly severe or extensive colonic inflammation, referred to as 'very early onset IBD' (VEO-IBD). The onset of IBD in this cohort of patients can occur within the first year of life, and these investigators studied single nucleotide polymorphisms (SNP) in subjects up to an age of 17 years. The researchers identified a *NOS2* (iNOS)-related SNP to be associated with two independent VEO-IBD clinical cohorts. Strong associations were observed with both VEO-ulcerative colitis and VEO-Crohn's disease. There was an association of the SNP with ulcerative colitis diagnosed between 11 and 17 years of age but no such association was observed in adult-onset IBD (over 17 years of age). Colonic biopsies from the VEO-IBD patients exhibited higher levels of staining for nitrotyrosine (suggestive of elevated NO production). The authors concluded that 'these studies suggest the importance of iNOS in genetic susceptibility to younger IBD presentation due to higher NO production' (Dhillon *et al.*, 2014).

Drug development: inflammatory bowel diseases

Mesalamine has been in use for over 5 decades as a front-line therapy for IBD; the mechanism through which mesalamine reduces mucosal injury in the GI tract remains unclear. Several anti-inflammatory activities have been proposed, including suppression of leukotriene synthesis, inhibition of IL-1 synthesis and scavenging of oxygen-derived free radicals and peroxynitrite (Abraham *et al.*, 2017). Alternatives to mesalamine are typically much more expensive and quite frequently associated with adverse effects (e.g. antibody-based therapies, **azathioprine** and corticosteroids). Thus, there is a need for more potent and safer drugs for treating IBD.

Given that NO has been shown to exert a wide range of protective, reparative and anti-inflammatory effects in the GI tract, we undertook to determine if an NO-releasing derivative of mesalamine would exhibit enhanced beneficial effects in animal models of colitis. One of the reasons that mesalamine is among the most commonly used drugs to treat IBD is that it is one of the safest of the many drugs used to treat this disorder (corticosteroids, azathioprine, monoclonal anti-TNF antibodies, etc.). However, mesalamine is only modestly effective in a significant proportion of patients. This lack of efficacy is largely due to the challenges of delivering sufficient amounts of mesalamine specifically to the sites of active inflammation in the GI tract. After administration, mesalamine is rapidly absorbed, but its beneficial effects are due primarily to topical actions at the sites of inflammation, while its adverse effects are largely due to systemic exposure. Delivery of mesalamine is particularly problematic for patients with Crohn's disease, which can occur anywhere along the length of the alimentary tract. Thus, various strategies have been employed to maximize the delivery of mesalamine to the site of inflammation, with mixed success.

Several years ago, we began developing a novel NO-releasing derivative of mesalamine for the treatment of IBD (Wallace *et al.*, 1999). Our focus subsequently changed to a **hydrogen sulfide** -releasing mesalamine derivative for several reasons (Chan and Wallace, 2013). Unlike the rationale behind NO-releasing NSAIDs, the NO-releasing mesalamine derivative was not developed to reduce toxicity (which is already quite low with mesalamine); rather, the objective was to broaden the range of anti-inflammatory activities and/or potency through the incorporation of an NO-releasing moiety.

In two rodent models of experimental colitis [trinitrobenzene sulfonic acid (TNBS) and the IL-10 knockout mouse] (Wallace *et al.*, 1999; Santucci *et al.*, 2005), the NO-releasing mesalamine derivative was substantially more effective than mesalamine in reducing disease severity (Figure 2). There was also a substantial reduction of granulocyte recruitment to the colon in rats treated with the NO-releasing mesalamine (as measured by tissue myeloperoxidase activity) (Figure 2). This was likely due in large part to the ability of the NO-releasing mesalamine derivative to inhibit leukocyte adherence to mesenteric post-capillary venules *in vivo* (Wallace *et al.*, 1999) (Figure 3). This was accompanied by significant reductions of colonic myeloperoxidase activity in the colonic tissue, consistent with enhanced anti-inflammatory activity

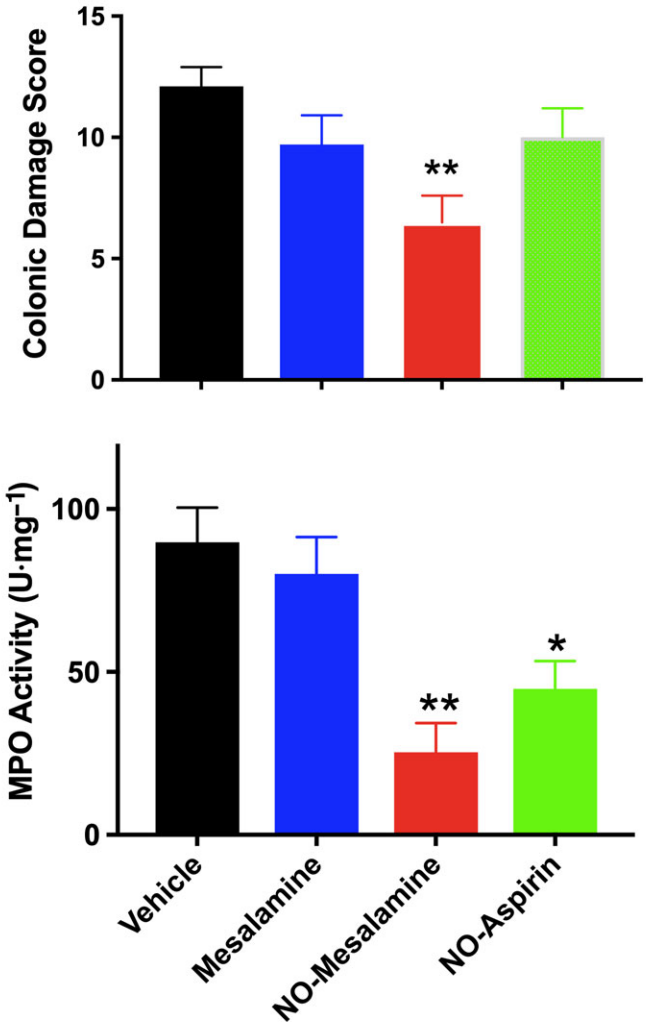

Figure 2

Effects of mesalamine (5-aminosalicylic acid), NO-releasing mesalamine and NO-releasing aspirin in a rat model of colitis. Rats treated with TNBS develop severe colitis (upper panel; n = 6 per group). Treatment with an NO-releasing derivative of mesalamine but not mesalamine itself significantly reduced the severity of colitis and the associated accumulation of granulocytes within the colonic mucosa [lower panel: measured as myeloperoxidase (MPO) activity]. The NO-releasing derivative of aspirin did not affect colonic damage severity but did significantly reduce colonic tissue MPO activity. *$P < 0.05$; **$P < 0.01$ as compared to the corresponding vehicle-treated group. All drugs were administered at 100 mg·kg^{-1}. This figure constructed from data reported in Wallace *et al.* (1999).

(Wallace *et al.*, 1999). The ability of NO-mesalamine to increase blood flow, as was apparent by the significant vasodilation of mesenteric venules in rats treated with this drug, may also have contributed to reduced tissue injury and/or accelerated repair observed with this mesalamine derivative (Wallace *et al.*, 1999). The improved efficacy with this novel compound may have also been in part attributable to an NO-dependent enhancement of inhibition of Th1 cell function and mucosal regulatory T-cell function (Santucci *et al.*, 2005). Selective induction of apoptosis of activated lamina propria Th1 cells, as well as induction of TGF- and IL-10-secreting cells by the NO-releasing mesalamine derivative resulted in significant attenuation of intestinal inflammation in the rodent models.

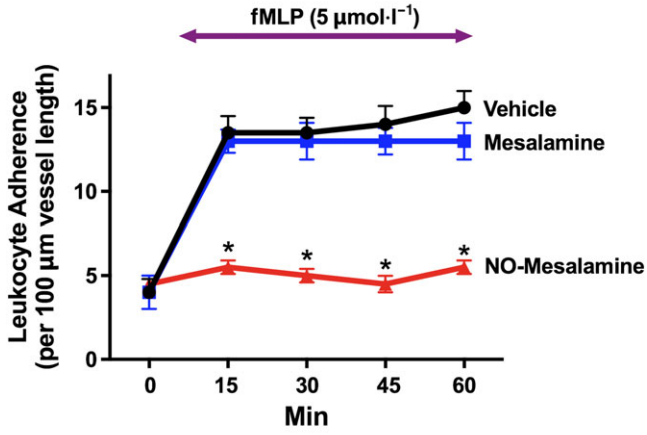

Figure 3

Effects of treatment with vehicle, mesalamine or NO-releasing mesalamine on leukocyte adherence to post-capillary mesenteric venules in rats before (time 0) and after superperfusion of the vessels with formyl-methionine-leucine-phenylalanine (fMLP; 5 µmol·L^{-1}). The test drugs were given at a dose of 100 mg·kg^{-1} p.o. 1 h before the start of the experiment. Results are expressed as mean ± SEM for six rats per group. *$P < 0.05$ versus vehicle-treated. Figure constructed from the data reported in Wallace *et al.* (1999).

NO, GI smooth muscle and visceral pain

The processing and absorption of food are dependent upon the coordinated contraction of the layers of smooth muscle along the length of the GI tract. Understanding muscle relaxation mechanisms is particularly important for understanding physiological processes such as sphincter relaxation, gastric accommodation and the descending peristaltic reflex (Gallego *et al.*, 2016). NO is one of the primary inhibitory neurotransmitters for GI muscular relaxation. It is synthesized through neuronal NOS (NOS1) enzyme activity, diffuses across the cell membrane to bind to its 'receptor' (guanylyl cyclase), activating several intracellular mechanisms that ultimately result in muscle relaxation.

Gastric accommodation is a response to the ingestion of food that involves relaxation of smooth muscle, allowing for distention of the stomach (particularly the fundus region of the stomach). Accommodation reduces the discomfort associated with distention and reduces the risk of food and gastric juice refluxing into the oesophagus. NO has been shown to play a key role in this process, as well as relaxation of smooth muscle in other parts of the GI tract. In 1990, NO was identified as an inhibitory NANC neurotransmitter (Bult *et al.*, 1990). The role of NO in gastric accommodation has been demonstrated by many groups, including Tack *et al.* (2002), who demonstrated that inhibition of NO synthesis with L-N^{G}-monomethyl arginine citrate impaired accommodation and enhanced meal-induced satiety. In humans, NO

has been shown to play important roles in maintaining basal tone of the fundus and in meal-induced satiety (Kuiken *et al*., 2002).

One of the most commonly occurring but poorly understood GI maladies is irritable bowel syndrome (IBS). It is referred to as a 'functional disorder' because the pathogenesis is poorly understood, and it is defined by a heterogeneous group of symptoms, such as cramping, bloating, abdominal pain, gas, diarrhoea or constipation or both. In contrast to the roles of NO in the GI physiological responses referred to above, NO also plays important roles in mediating smooth muscle dysfunction and dysfunction of the afferent pathways that lead to visceral hypersensitivity and pain. Considerable research has been focused on identifying mechanisms through which NO may mediate some of the functional changes that characterize IBS and that contribute to symptom generation.

Transient receptor potential cation channels, such as **TRPV4**, play important roles in intestinal inflammation and visceral pain. NO was recently shown to be a key mediator of these interactions, primarily *via* nNOS, both *in vitro* and *in vivo* (Fichna *et al*., 2015). TRPV4 activation resulted in decreased motility as a consequence of a reduction in NO-dependent calcium release from enteric neurons. This was demonstrated both in humans and in mice. Further evidence for role of NO in mediating visceral pain was provided by Kuiken *et al*. (2006). They studied 12 patients with IBS (documented hypersensitivity to rectal distension) and 10 healthy controls. The effects of placebo versus an NOS inhibitor (N^G-monomethyl-L-arginine; L-NMMA) on rectal sensitivity to distension, rectal compliance and resting volume were evaluated in a double-blind, randomized, cross-over manner. **L-nitroarginine methyl ester (L-NAME**) did not alter resting volumes or rectal compliance in healthy volunteers or in IBS patients. However, administration of L-NMMA significantly increased the threshold for discomfort/pain in the IBS patients, suggesting that NO is involved in the pathophysiology of visceral hypersensitivity (Kuiken *et al*., 2006).

Animal models of IBS do not fully replicate the clinical features in humans (which can be highly variable from patient to patient), but there are good models for assessing visceral pain and motor dysfunction. One such animal model used to study the pathophysiology of pain is the **neutral endopeptidase** (NEP)-deficient mouse. NEP is an endopeptidase that can cleave a range of substrates, including **substance P**, enkephalins and **bradykinin**. The NEP-deficient mice exhibit hyperalgesia in several animal models, at least in part related to activation of bradykinin B_2 receptors (Fischer *et al*., 2002). Inhibition of NO synthesis with L-NAME reduced pain in these models.

Another model that has been employed in an attempt to mimic IBS-like symptoms is the neonatal maternal separated rat (Tjoing *et al*., 2011). In this study, rats were subjected to 3 h of maternal separation on postnatal days 2 through 21. As compared to the controls, the maternal separated rats exhibited significantly higher levels of NO production and marked increases in the expression of iNOS but not nNOS or eNOS. The vehicle-treated rats subjected to neonatal maternal separation exhibited a significantly lower pain threshold and a marked increase in EMG activity in response to colonic distension, as compared to the maternal separated rats. Treatment of the latter either of two non-selective NOS inhibitors increased pain threshold pressure and attenuated electromyographic activity in the maternally-separated rats. Unfortunately, this study did not include the use of selective inhibitors of the different NOS enzymes (Tjong *et al*., 2011). Nevertheless, this study does suggest that an elevated production of NO, most likely from iNOS, contributes significantly to the development of increased visceral sensitivity, consistent with other animal studies described above and consistent with the hypothesis that iNOS similarly contributes to the generation of IBS-related symptoms in humans.

Drug discovery: irritable bowel syndrome

There are few effective therapeutic options for the treatment of IBS, largely because of the wide range of symptoms and the high variability of those symptoms from patient to patient. Trimebutine is a relatively weak opioid receptor agonist that has been used to treat the pain and hypermotility that is common with IBS. Distrutti *et al*. (2009) described the effects of a novel NO-releasing trimebutine derivative, which was assessed both in healthy rats and in rats with post-colitis hypersensitivity. Pain was assessed by the abdominal withdrawal response to colorectal distention (CRD). The post-colitis hypersensitivity studies were performed 4 weeks after induction of colitis with TNBS. Importantly, this was a point in time when healing of the colitis was complete, but significant alterations in visceral pain sensitivity remained. Treatment with the NO-releasing trimebutine derivative resulted in a dose-dependent reduction in CRD-induced nociception, significantly greater than was observed with trimebutine itself (Distrutti *et al*., 2009). As well as the functional readout of pain, spinal expression of cFOS mRNA was significantly reduced by the NO-releasing trimebutine derivative but not by trimebutine. The beneficial effects of the NO-releasing trimebutine derivative were blocked by the administration of an opioid antagonist or methylene blue (a NO scavenger) but not by an inhibitor of NOS (L-NAME). In addition, the expression of several genes involved in inflammation and pain, such as COX-2, **IL-1β**, TNFα and iNOS, were up-regulated in colonic tissue from the post-colitis rats as compared to controls. Treatment with the NO-releasing trimebutine derivative, but not with trimebutine, reversed these effects (Distrutti *et al*., 2009).

Development of this drug does not appear to have continued after the publication from Distrutti *et al*. (2009). A hydrogen sulfide-releasing derivative of trimebutine was developed by Antibe Therapeutics and was licensed to GiCare Pharma several years ago. In preclinical studies, this compound (GIC-1001) was shown to be antinociceptive in mice and to exert both peripheral opioid agonistic activity and have the ability to release hydrogen sulfide (Cenac *et al.,* 2016). It progressed to phase 2 clinical trials, but the increase in analgesic activity versus trimebutine was insufficient to warrant further development.

Conclusions

NSAID-gastroenteropathy, IBD and IBS are among the most common disorders affecting the GI tract. NO has been

demonstrated to contribute to the pathogenesis of each of these disorders, and in each case, NO or an inhibitor of NO synthesis has been proposed as a treatment. Thus far, however, NO-based therapies for these disorders have not been successfully translated to the clinic or the market. NO-releasing NSAIDs progressed to substantive phase 3 clinical studies, but the regulatory (and associated financial) hurdles in the era of selective COX-2 inhibitors were deemed as insurmountable. As we have reported previously (Wallace *et al.*, 2018), the development of safer and/or more effective drugs for GI disorders may be more successful when the 'parent drug' is covalently linked to a hydrogen sulfide-releasing moiety, rather than to a NO-releasing moiety. One such drug has now progressed through phase 2 efficacy and toxicity clinical trials, with promising results (Wallace *et al.*, 2018).

Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding *et al.*, 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander *et al.*, 2017a,b).

Conflict of interest

The author declares no conflicts of interest.

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