Antagonism of protease activated receptor 2 protects against experimental colitis

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Running title: PAR2 antagonism in colitis

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Abbreviations; IBD; Inflammatory bowel disease. IBS; irritable bowel syndrome. PAR2; protease-activated receptor-2. TNBS; 2,4,6 trinitrobenzenesulfonic acid. SLIGRL-NH₂; PAR2 agonist peptide. 2Furoyl-LIGRLO-NH₂; PAR2 agonist peptide.

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Abstract

Many trypsin-like serine proteases like β-tryptase are involved in the pathogenesis of colitis and inflammatory bowel diseases. Inhibitors of individual proteases show limited efficacy in treating such conditions, but also likely disrupt digestive and defensive functions of proteases. Here we investigate whether masking their common target, protease activated receptor 2 (PAR2), is an effective therapeutic strategy for treating acute and chronic experimental colitis in rats. A novel PAR2 antagonist (GB88) was evaluated for blockade of intracellular calcium release in colonocytes, and for anti-inflammatory activity in acute (PAR2 agonist–induced) versus chronic (TNBS–induced) models of colitis in Wistar rats. Disease progression (disease activity index, weight loss, mortality) and post-mortem colonic histopathology (inflammation, bowel wall thickness, myeloperoxidase) were measured. PAR2 and tryptase co-localization was investigated using immunohistochemistry. GB88 was a more potent antagonist of PAR2 activation in colonocytes than a reported compound ENMD-1068 (IC₅₀ 8 µM vs 5 mM). Acute colonic inflammation induced in rats by PAR2 agonist SLIGRL-NH₂ was inhibited by oral administration of GB88 (10 mg/kg), with markedly reduced oedema, mucin depletion, PAR2 receptor internalisation and mastocytosis. Chronic TNBS–induced colitis in rats was ameliorated by GB88 (10mg/kg/day/p.o.), which reduced mortality and pathology (including colon obstruction, ulceration, wall thickness, myeloperoxidase release) more effectively than the clinically used drug sulfasalazine (100 mg/kg/day/p.o.). These disease-modifying properties for the PAR2 antagonist in both acute and chronic experimental colitis strongly support a pathogenic role for PAR2 and PAR2–activating proteases, and therapeutic potential for PAR2 antagonism, in inflammatory diseases of the colon.
Introduction

Crohn’s disease (CD) and ulcerative colitis (UC) are common forms of chronic inflammatory bowel disease (IBD) that share common pathologies. (Gearry et al., 2010) UC affects the colon and rectum, CD affects multiple regions of the colon and ileum, and each condition has characteristic patterns of ulcerative mucosa. IBDs increase the risks of developing sepsis following stenosis–related bowel perforation, (Andersson and Soderholm, 2009) colorectal carcinoma, (Laukoetter et al., 2011) and multiple organ dysfunction. Tissue biopsies and colonic luminal fluid from human IBD patients and from animals with experimental colitis show high expression levels of serine protease enzymes, such as trypsin, tryptase and other trypsinogens. (He and Xie, 2004; Hansen et al., 2005; Kawabata et al., 2008; Lee et al., 2010) These enzymes have well known protein–digesting and gastrointestinal properties in the gut, but their roles in the pathogenesis of IBD have been very difficult to elucidate due to their pleiotropic functions in vivo. Clinical and experimental studies have shown broad–spectrum and more specific protease inhibitors, such nafamostat mesilate, have been used with some efficacy in relieving colitis–like symptoms, (Isozaki et al., 2006; Lee et al., 2010) however this may be limited due to the likely involvement of multiple proteases in IBDs.

Serine proteases (e.g. trypsin, β-tryptase) are now known to activate intracellular signaling pathways through a G protein–coupled receptor, protease–activated receptor 2 (PAR2). (Macfarlane et al., 2001) There are no known endogenous non–proteolytic agonists. (Cocks and Moffatt, 2000) PAR2 is
ubiquitously expressed on gastrointestinal mucosa, (Cocks et al., 1999; Cenac et al., 2002) smooth muscle, (Cocks et al., 1999) neurons (Fiorucci and Distrutti, 2002) and many cell types exposed to serine proteases. (Kawabata et al., 2008) Precise roles for protease-mediated PAR2 signalling in the gut are not defined, but PAR2 and its activating proteases are important in the GI tract, (Kawabata et al., 2008; Browning, 2010) and there is evidence of a pathogenic role for PAR2 in CD, UC (He et al., 2004; Hansen et al., 2005; Browning, 2010) and irritable bowel syndrome (IBS). (He, 2004; Barbara and Cremon, 2008; Lee et al., 2010) PAR2 is up-regulated on various cell types in colonic tissue taken from IBD and IBS patients. (Cenac et al., 2002; Kawabata et al., 2008; Lee et al., 2010) PAR2 activation, possibly via neurogenic mechanisms (Fiorucci and Distrutti, 2002), induces colonic motility changes (Cocks et al., 1999) chloride–mediated increases in colonic permeability, oedema, (Cenac et al., 2002) leading to diapedesis and bacterial translocation across the protective mucosa (Roka et al., 2007; Lee et al., 2010). Furthermore, intracolonic PAR2 activation (using trypsin, tryptase or the peptide SLIGRL-NH₂) results in mucosal damage, bowel wall thickening and myeloperoxidase (MPO) activity, which do not occur in PAR2⁻/⁻ mice. (Cenac et al., 2002; Patel and Shah, 2010) PAR2⁻/⁻ mice also do not develop most symptoms of TNBS-⁻ (Hyun et al., 2008) or bacteria-⁻ induced colitis. (Hansen et al., 2005; Cottrell et al., 2007) An efficacious PAR2 antagonist could help to clarify in vivo roles for PAR2 and its activating proteases in the aetiology of IBD, and validate a new therapeutic avenue for treating IBD.
We recently disclosed the first potent, selective and orally active PAR2 antagonists, (Barry et al., 2010; Suen et al., 2011) the only effective compounds known to inhibit PAR2 activation by endogenous proteases, synthetic peptides and non−peptide agonists. Here we evaluate the efficacy of one such antagonist (GB88, 10 mg/kg/day p.o.) in rat models of acute and chronic inflammatory bowel disease induced by a PAR2 agonist (SLIGRL-NH₂) or 2,4,6 trinitrobenzenesulfonic acid (TNBS), respectively. GB88 was effective in ameliorating IBD−like symptoms in both rat models, much more effective than the clinically used sulfasalazine (100 mg/kg/day p.o.) in TNBS-induced colitis. These results validate the importance of PAR2 to pathology in experimental colitis, (Cottrell et al., 2007; Kawabata et al., 2008) and verifies PAR2 antagonism as an effective strategy for treating IBDs.

METHODS

Animals.

Male Wistar rats (aged 8-9 weeks, 250-300 g), bred and housed at the Australian Institute for Bioengineering and Nanotechnology were maintained in a 12 h light/dark cycle with food and water provided. Experiments were approved by the animal ethics committee of The University of Queensland (Australia).

Drugs, peptides and chemicals.

Picrylsulfonic acid (2,4,6-trinitrobenzenesulfonic acid (TNBS)) and non-steroidal anti-inflammatory drug sulfasalazine were supplied by Sigma-Aldrich (Australia). PAR2 activating peptides SLIGRL-NH₂ and 2Furoyl-LIGRLO-NH₂ were
synthesised by standard solid phase peptide chemistry. PAR2 antagonists GB88 (Suen et al., 2011) and ENMD-1068 (Kelso et al., 2006) were synthesised by standard solution phase chemistry following literature procedures. (Barry et al., 2010) All compounds were purified by high performance liquid chromatography methods. GB88 and ENMD-1068 were characterised by proton nuclear magnetic resonance spectroscopy. GB88 and sulfasalazine were dissolved in olive oil (generic brand) and administered by oral gavage (p.o. polypropylene feeding tubes, 18 G x 75 mm, Instech Solomon, Aust, ≤500 μL).

Cell assay.

Human colorectal adenocarcinoma (HT29) cells were incubated with a calcium binding dye (Fura3) buffer for 1 h at 37°C. Cells were treated with GB88 or a previously reported compound (ENMD-1068) at various concentrations for 15 min prior to addition of 2Furoyl-LIGRLO-NH2 (1 μM). Intracellular calcium mobilization was measured by differences in fluorescence plotted against corresponding antagonist concentrations, enabling determination of antagonist potency.

PAR2−induced acute colonic inflammation.

Methods were based on previous descriptions, (Cenac et al., 2002) modified for rats. Rats were fasted overnight, anaesthetised with isofluorane 2 h after receiving either a single dose of oral GB88 (10 mg/kg p.o.) or vehicle (olive oil). A polyethylene catheter (1.7 mm outer-diameter) was inserted 8 cm into the colon via the anus, through which SLIGRL-NH2 (1 mg/rat) or saline vehicle (∼500 μL) was administered. Rats remained anaesthetized for 10 min and maintained at an angle of 40° to prevent leakage from the anus. Rats were allowed to recover with
food and water supplied. All were terminated at 10 h post-induction, and scored appropriately for disease activity index and macroscopic disease (see disease activity index below). Colon tissue was taken for wet:dry ratio and MPO assay.

**TNBS–induced chronic colitis model.**

Methods were based on those previously described. (Fiorucci et al., 2001) On Day 0, rats were weighed, given respective treatments of either sulfasalazine (100 mg/kg in olive oil p.o.), (Deng et al., 2009) GB88 (10 mg/kg in olive oil) or vehicle (500 μL olive oil p.o.) and fasted for 24 h with water supplied *ad libitum*. The following day (Day 1), rats were weighed, dosed with respective compound and anaesthetised with isofluorane. A polyethylene catheter was inserted intrarectally (8cm). Control and drug-treated animals received an intracolonic dose of TNBS (80 mg/kg in saline and 250 μL, 50% ethanol in water (Fiorucci et al., 2001)). Sham animals received vehicle only, with TNBS replaced by equivolume water. Rats were maintained in an inclined position (≈40°) for 30 min, then allowed to recover with food and water supplied. Rats were dosed daily with the respective compound, weighed and scored for general health and disease progression. All rats were sacrificed on day 8 of experimentation unless they were sacrificed prior.

**Disease activity index (DAI).**

DAI for both acute and chronic models was assessed and scored by an expert blinded to treatments. Scores incorporated mobility, gastro-intestinal pathology, discomfort and generalised sickness behaviour criteria (each scored 0–3). Any animal that reached stage 3 in any criteria was euthanised by CO₂ inhalation, as
stipulated in the ethical agreement, and recorded as a disease-related mortality. Scores were summed and expressed as total disease score (max. 12). DAI was measured at end point only (10 h) in the acute study, and daily in the chronic TNBS study (see Supplemental Methods for more information).

**Macroscopic disease index.**

Colons were dissected postmortem and given a macroscopic disease score based on that previously described (Bobin-Dubigeon et al., 2001) with some minor alterations (maximal score = 14, see Supplemental Table 1). The entire colon length was removed between the ileo–colic and colo–rectal junction. Length and maximal distension width were measured. Sections of affected colon were taken for biochemical analysis, histology and wet:dry weight (see Supplemental Methods for more information).

**Histology and immunohistochemistry.**

Tissue samples were embedded in paraffin wax, cut on a microtome (5 μm) and stained with either Haematoxylin and Eosin (H&E) or alcian blue (pH 1.0) and safranin–O using standard protocols. For immunohistochemistry, tissue was labeled with antibodies raised against PAR2 (N19 1:100, Santa Cruz) and/or mast cell tryptase (AA1, 1:100, Abcam) using standard 10mM citrate antigen retrieval and 4h (primary) incubation protocols. All fluorescence images were subject to background subtraction and brightness enhancement using standard ImageJ algorithms to clarify staining patterns (see Supplemental Methods for more information).

**Bowel wall thickness**
Bowel wall thickness was measured by a researcher blinded to treatments. Measurements were made from H&E–stained photomicrographs (20× lens) using ImageJ software (1.42q). Image pixels were calibrated into micron (0.143 pixels/μm) and distance from outer circular muscle to the inner crypt base measured.

**Myeloperoxidase and ELISA Assay.**

Unfixed colon sections were homogenized in 4°C 0.5% hexadecyl trimethylammonium bromide/PBS buffer (100 mg/mL w/v, pH=6.0) and centrifuged at 13K rpm for 10 min at 4°C. Supernatant (100 μL) was transferred (in duplicate) to a 96 well plate to which σ-Dianisidine (20 μL, 2.85 mg/mL in 1% H₂O₂/PBS) was added and mixed by aspiration. The plate was allowed to incubate for 15 min at room temperature (in dark) then transferred to a fluorimeter (FLUOstar optima). Absorbance was read at l = 450 nm. Data was expressed as absolute optical density units. Cytokine expression (TNFα, IL-6) in tissue homogenates was measured using ELISA (BD Bioscience) according to the manufacturer’s instructions.

**Data analysis.**

Experimental results were expressed as mean ± SEM. Data were analysed using Prism software (v5.0a, GraphPad Software, San Diego, CA). Statistical comparisons were performed using two-way repeated measures ANOVA for temporal data sets involving three or more groups. For individual time points, data was analysed with one-way ANOVA and groups compared with Bonferroni planned comparisons. Significance was set at p<0.05.
Results

**GB88 is a PAR2 antagonist in colon cells.**

GB88 was a potent antagonist of intracellular Ca\(^{2+}\) release induced in HT29 adenocarcinoma cells by PAR2 agonist 2Furoyl-LIGRLO-NH\(_2\) (IC\(_{50}\) 8 µM, Fig. 1, (Suen et al., 2011)). This *in vitro* antagonist potency is similar to what we found in other cell types (IC\(_{50}\) 2 -5µM), when we also showed that GB88 was specific for PAR2 over PAR1 *in vitro* and in a rat paw oedema assay.(Suen et al., 2011) Moreover, here we show that GB88 was also three orders of magnitude more potent than ENMD-1068 (IC\(_{50}\) 5 mM; Figure 1), a compound previously reported to be an antagonist of PAR2, albeit only at millimolar concentrations.(Kelso et al., 2006)

**PAR2-induced colonic inflammation.**

Intracolonic administration of the hexapeptide PAR2 agonist, SLIGRL-NH\(_2\) (1 mg/rat, n=6), caused distinct colonic inflammatory symptoms in rats, as similarly reported by others in mice.(Cenac et al., 2002) After 10 h, rats displayed a mild disease activity index (DAI) (diarrhoea, chromodacryorrhoea, reduced grooming and mobility) compared to sham (n=5) as previously reported(Cenac et al., 2002). Post-mortem analysis confirmed that SLIGRL-NH\(_2\) induced mild colonic hyperaemia and bowel wall thickening due to oedema. Microscopically, colons showed distinct increases in wall thickness, and histopathological scores (p>0.05, Fig. 2A and B respectively). Wet:dry weight ratio was also significantly increased (p>0.05, Fig. 2C). Prophylactic GB88 (10 mg/kg p.o., n=5) prevented the SLIGRL-NH\(_2\) induced wall thickness changes, histopathology, and oedema.
(wet:dry weight; p>0.05, Fig. 2A-C). In H&E stained sections, little diapedesis of leukocytes was however observed in the colon wall of rats treated only with SLIGRL-NH₂ (Supplemental Fig. 1). Similarly, no changes in MPO expression in tissue homogenates were observed (Supplemental fig. 1). These data confirm that PAR2 activation promotes colonic oedema with little leukocyte diapedesis.(Cenac et al., 2002)

The mast cell population almost doubled in the submucosa of SLIGRL-NH₂−treated rats and was prevented by pre−treatment with GB88 (Fig. 2D, p<0.05). The percentage of mast cell degranulation increased in rats given SLIGRL-NH₂ alone, although this did not reach statistical significance (p=0.06, Fig. 2E). Distal colonic mucosal goblet cells of rats treated with SLIGRL-NH₂ also showed a reduction in alcian blue (mucin) positive cells in the mucosa (p<0.05, Fig. 3A), similar to that observed in other models of experimental colitis-like inflammation(Linden et al., 2008). The PAR2 antagonist GB88 prevented these changes in mucin expression (Fig. 3A upper panels, B).

PAR2 immunofluorescence was located in colonic mucosa, particularly lining the epithelium, but also in the membrane and soma of goblet cells (Fig. 3A lower panels). The expression of PAR2 became more intense in the soma of goblet cells and less on the membrane at 10 h following SLIGRL-NH₂ treatment, suggesting activation of PAR2 causes its internalisation in goblet cells (Fig. 3A lower panels, C). This was prevented by GB88 (p<0.05). Regression analysis revealed a strong negative correlation between mucin staining and PAR2 staining in the soma of goblet cells (Pearson r=-0.79, p<0.05, Fig. 3D). These data
suggest that activated PAR2 is internalised in goblet cells in which mucin levels are depleted. Such effects on mucin may contribute to disease. (Linden et al., 2008).

Tryptase positive cells (likely mast cells) were found in the lamina propria and mucosal crypts. There was also a pericellular staining pattern in the mucosal crypts, which likely represents extracellular tryptase surrounding goblet cells, since it co-localized with PAR2 immunoreactivity (Fig. 4). Due to paucity of staining, tryptase levels could not be accurately analysed between treatment groups. There was, however, little evidence to suggest that tryptase–positive colonic mast cells had any recognisable PAR2 immunoreactivity (Fig. 4).

**TNBS–induced colonic disease progression.**

GB88 was further examined in a more non-specific and chronic model of colitis. Rats treated with TNBS, but not GB88 (TNBS–control), showed a significant rapid deterioration in DAI on Day 2. This initial worsening of symptoms occurred in all TNBS–control animals, regardless of oral treatments given (Fig. 5A). Sham animals not receiving TNBS displayed mild diarrhoea, but recovered by Day 4. These symptoms likely result from the ethanol vehicle routinely used in the induction phase in this assay. TNBS–treated control animals had a DAI that progressively deteriorated (Figure 5A), which evidently was terminal (Fig. 5C). Intestinal symptoms in this group consisted of a palpable abdominal mass, evident in 7 of 11 control animals. In all groups body weight deteriorated until Day 3, following which weight began to increase, returning to baseline levels in all but TNBS–control animals (Fig 5B). Such weight re-gain is artefactual due to the
clear bowel obstruction evident in this group. Mortality in the TNBS−control group reached 55% by Day 8 (Fig 5C).

GB88−treated (10 mg/kg/day p.o.) animals showed similar disease profiles to those treated with the colitis drug, sulfasalazine (100 mg/kg/day p.o.). Both GB88 and sulfasalazine treatments caused marked improvement in DAI from Day 2 onwards (Fig. 5A), as compared to TNBS−controls, exceeding baseline weights at end point. GB88−treated rats showed an almost complete recovery in DAI. An abdominal mass was noted in only 1 of 11 and 3 of 12 animals in the GB88 and sulfasalazine−treated groups, respectively. However, GB88 was much more effective than sulfasalazine in preventing TNBS−related mortality (8.3% vs 33.3% mortality, respectively, p<0.05 Figure 5C).

**Macroscopic pathology.**

Colonic disease state was evident in a reduced colonic length (81% of sham, p<0.05, Fig. 6A)(Glauben et al., 2008) and distension increase (155% than sham, p<0.05, Fig. 6B). Macroscopic analysis of colon dissected from TNBS−controls had significant pathology, including abdominal adhesions (Fig. 6D), hyperaemia, and usually continuous ulceration and patchy necrotic lesions (Fig. 6C). Bowel obstruction was confirmed in 64% (7/11) of TNBS−controls (Fig. 6E). Both GB88 and sulfasalazine treatments imparted significant improvements in colonic length, distension, and other macroscopic pathologies such as reduced colonic adhesions, ulceration and necrosis (p<0.05, Fig. 6A−D). Both treatments were effective at reducing incidence of macroscopic disease symptoms, particularly bowel obstruction (p<0.05, Fig. 6E).
Oedema.

Colons taken from TNBS−controls had significantly greater wet:dry ratios than shams, confirming oedema (p<0.05, Fig. 7A). Even though not returned to sham levels, both GB88 and sulfasalazine treatments reduced wet:dry ratio, however only GB88 showed statistical significance (p<0.05 Fig. 7A). In agreement with colonic water content, TNBS−induced MPO activity was ameliorated by GB88 treatment (p<0.05), whereas sulfasalazine showed little benefit (p>0.05. Fig. 7B).

Histopathology.

H&E stained colon sections from TNBS−control animals showed extensive cardinal colitis−like tissue damage, (haemorrhage, transmural ischaemia and necrosis (Fig. 8)), symptoms synonymous with human (non−specific) IBD−like symptoms. Histopathological scoring of H&E sections confirmed that both GB88 and sulfasalazine imparted substantial improvements in colonic health, showing reductions in mucosal ulceration, oedema and diapedesis in all tissue layers compared to TNBS−controls (Fig. 9A−C, respectively). Bowel walls were thickest in TNBS−controls, which were normalised to sham levels by GB88 (p<0.05, Fig. 9D). The effects of GB88 were consistently better than sulfasalazine in all aspects of experimental colitis tissue health, however, differences did not reach statistical significance. These results correlate with the colonic water content and MPO concentrations, which in combination indicate that GB88 effectively reduced both oedema and diapedesis. All TNBS−treated colons were sectioned and stained for mast cells and mucin using alcian blue/safranin-O, however due to the advanced necrotic state of colic tissue taken from TNBS−control animals, viable
cells could not be accurately counted in these tissue. The advanced necrotic state of the tissue also influenced cytokine expression, as TNBS–control colon homogenates had unexpectedly low levels of cytokines (TNFα and IL-6) using ELISA compared to treatment groups (Supplemental fig. 2).

Discussion

The results demonstrate that the PAR2 antagonist GB88 has significant disease-modifying benefits in experimental colitis, supporting a pivotal role for PAR2 in the pathogenesis of IBD. Until now, there has been no potent or orally bioavailable PAR2 antagonist available to investigate PAR2 antagonism in vivo. We have found here that a PAR2 agonist induces acute symptoms of colitis-like inflammation (oedema, mastocytosis, mucin depletion, mucosal erosion) in rats, and demonstrated that the PAR2 antagonist GB88 ameliorates colonic inflammation in this model. Moreover, GB88 was also efficacious in a more general chronic (TNBS-induced) model of experimental colitis in rats, being more effective than the clinically used sulfasalazine (at 10% of the dose). The data support the development of PAR2 antagonists for treating IBDs.

In rats receiving intracolonic PAR2 agonist, we have also made the novel finding of an inverse relationship between PAR2 expression in the cytosol of mucosal goblet cells and the expression of mucin in the colon mucosa. PAR2 activation caused mucin depletion from colon mucosa. This suggests that PAR2 agonists may induce a long lasting (at least 10 h) receptor internalisation accompanied by mucin depletion in colonic goblet cells. PAR2 agonists induce intracellular calcium release in colonocytes, and this is known to be a stimulus for
mucin secretion from goblet cells.(Davis, 2002) Mucin depletion is often associated with IBD-like disease,(Linden et al., 2008) and may limit the effectiveness of the mucous barrier to bacterial infection to the colon mucosa which, together with increased colonic permeability, may allow bacterial translocation to the colon wall and further contribute to the protease–mediated disease.

There are many sources of PAR2-activating proteases in the colon, including resident cells (i.e. mast cells), infectious pathogenic flora (Escherichia Coli, Citrobacter rodentium, Clostridium difficile),(Hansen et al., 2005; Cottrell et al., 2007) and allergen–derived proteases (cockroach, dust mite).(Shpacovitch et al., 2008; Day et al., 2010) While concentrations of PAR2-activating serine proteases (i.e. trypsin, β-tryptase) increase in colonic luminal fluid in human and animal IBDs,(He and Xie, 2004; Hansen et al., 2005; Kawabata et al., 2008; Lee et al., 2010) recent evidence suggests that pathogenic PAR2–activating proteases may come from within the colon wall itself(Lau et al., 2010) and not the lumen.(Barbara and Cremon, 2008) PAR2 expressed in specific pools on the basolateral, but not apical, membrane of a colon cell monolayer are responsible for zonula occludens-1 related tight junction reorganisation causing the described colonic permeability changes.(Lau et al., 2010)

Mast cells are resident in the colon wall during IBD–like inflammatory events and produce the PAR2 agonist protease, β-tryptase.(He et al., 2004) As also shown here, mastocytosis and degranulation is associated with IBD-like conditions.(He, 2004; Barbara and Cremon, 2008; Hamilton et al., 2011) Since
PAR2 is responsible for a mild form of colonic inflammation, it seems likely that degranulating mast cells produce β–tryptase (Hamilton et al., 2011) that causes the PAR2 mediated colitis symptoms (hyperaemia, oedema, mastocytosis, mucin depletion) observed here. This is supported by our observations of co-localization of PAR2 and tryptase expression in and around goblet cells, the induced mucin depletion within these cells, and the blockade of inflammation, mucin depletion and mastocytosis by PAR2 antagonist GB88.

Various serine proteases and their precursors (e.g. trypsinogens (Hansen et al., 2005; Cottrell et al., 2007) and tryptases (He et al., 2004; He and Xie, 2004)) released by cells resident in the colon may have antibacterial roles (Huang et al., 2001; Thakurdas et al., 2007). These endogenous antibiotics could contribute to the primary barrier protection of the gut. Thus, although broad–spectrum and more specific protease inhibitors, like nafamostat mesilate, have been used to manage both human and animal IBD–like diseases with some success, (Lee et al., 2010) these likely also disrupt normal digestive and defensive roles for proteases. (Huang et al., 2001; Thakurdas et al., 2007) For this reason, antagonists of PAR2, such as GB88, may provide better therapeutic value than protease inhibitors or common anti-inflammatory treatments, such as sulfasalazine, in IBDs.

Since we find little evidence of PAR2 expressed on β–tryptase–containing mast cells in our immunohistochemistry study, perhaps the reported mast cell degranulation is not directly due to PAR2 activation on mast cells, but possibly governed by PAR2 on sensory nerve endings, inducing a neurogenic
inflammatory response. (Chen et al., 2009) In such a case, neuropeptides (i.e. Substance P, VIP) released from PAR2–activated c-fibres may cause a mast cell degranulation leading to inflammation. (Costa et al., 2008) Certainly, mast cells are often seen clustered around nerve endings. (Lam and Schmidt, 2010) Whether such PAR2–induced neurogenic mast cell degranulation exists needs to be further determined in order to fully understand an apparently delicate relationship between protective and disease-causing properties of PAR2.

In conclusion, we have shown that a PAR2 antagonist is protective in both SLIGRL-NH₂– and TNBS- induced experimental colitis in rats. Our results link the pathogenesis of PAR2 agonist-induced colonic inflammation with mastocytosis and co–expression of β-tryptase and PAR2 around mucin-depleted goblet cells. Importantly, β-tryptase has recently been linked to colitis through a gene depletion study (Hamilton et al., 2011). The current results connect PAR2 activation to β-tryptase release and mast cells in the pathogenesis of TNBS colitis, and demonstrate how PAR2–activating proteases can initiate or propagate colonic inflammation. Our data highlight PAR2 antagonism as a potential new therapeutic strategy for treating human chronic inflammatory bowel diseases.

Author contributions;

Participated in research design: Lohman, Cotterell, Suen, Liu, Vesey and Fairlie.

Conducted experiments: Lohman, Cotterell, Suen and Do.

Contributed new reagents or analytic tools: Liu and Vesey

Performed data analysis: Lohman, Cotterell and Suen
Wrote or contributed to the writing of the manuscript: Lohman and Fairlie
References


Footnotes

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Figure legends

Figure 1. GB88 is a potent antagonist of Ca$^{2+}$ mobilization in HT29 colonocytes. Intracellular Ca$^{2+}$ release in HT29 colonocytes induced by 2f-LIGRLO-NH$_2$ (1.0 μM) in the presence of varying concentrations of GB88. GB88 was 3 orders of magnitude more potent as a PAR2 antagonist than a reported compound ENMD-1068.

Figure 2. PAR2-induced colonic inflammation. Intracolonic administration of SLIGRL-NH$_2$ (1 mg/rat) induced (A) an increased bowel wall thickness (*p<0.05 from sham, n=6, ANOVA planned comparison), (B) a mild but significant colonic inflammation (**p<0.01 from sham, n=5) and, (C) an increased wet:dry ratio (*p<0.05 from sham). Pre-treatment with GB88 (10 mg/kg p.o., n=5) prevented colonic pathology, wall thickness and wet:dry ratio changes. (D) Mast cell population doubled in lamina propria of rats given SLIGRL-NH$_2$ (*p<0.05 from sham, ANOVA planned comparison), which was prevented by GB88. (E) Active/degranulated mast cells were found in higher numbers in submucosa of rats given SLIGRL-NH$_2$, as determined by Alcian blue/Safranin-O staining, but this was not statistically significant (p=0.06).

Figure 3. PAR2-induced colitis is associated with mucosa mucin depletion. (A) SLIGRL-NH$_2$ clearly caused mucin depletion from colon mucosa. Upper panels: representative images mucin positive cells (blue) and mucin depleted cells (red)). Lower panels: representative images of PAR2 (N19) stained colonic
mucosa, showing PAR2 translocation from membrane to cytoplasm following SLIGRL-NH₂ treatment. Note that images are not serial sections. (B) Quantitation of goblet cells containing mucin-like staining (blue) expressed as % of total cells (sum of red and blue, *p<0.05 from sham, ANOVA planned comparison). (C) Quantitation of PAR2-like immunofluorescence in goblet cells with either membrane or cytosolic staining (expressed as % total cells). SLIGRL-NH₂ clearly causes receptor internalisation that is prevented by GB88 (**p<0.01 from sham, ANOVA planned comparison). (D) Regression analysis revealed a strong negative correlation (Pearson r = -0.79, p<0.05) between the goblet cell mucin content (from B) and cytosolic PAR2 expression (from C), suggesting that mucin is depleted when PAR2 is internalized in goblet cells.

**Figure 4. PAR2 and tryptase co-localise in colonic mucosa.** (A) PAR2 immunoreactivity (red) shows PAR2 is distributed throughout goblet cells. Tryptase (green) expression appears pericellular to goblet cells expressing PAR2, suggesting tryptase is located around these PAR2-expressing cells. (B) No distinct PAR2-like immunoreactivity was observed in tryptase-positive mast cells (Arrows, image is digital zoom of region outlined by circle in merged image A). (C) Absence of primary antibodies in negative controls shows no specific immunoreactivity.

**Figure 5. GB88 reduces TNBS-induced disease-like symptoms.** Both GB88 (10 mg/kg/day p.o., n=11) and sulfasalazine (100 mg/kg/day p.o., n=12)
treatments showed distinct improvements in (A) disease activity index (DAI, *p<0.05, **p<0.01, ***p<0.001 from sham, n=10, ANOVA 2-way repeated measures planned comparison) and (B) weight re-gain as observed in TNBS–control rats (n=11, *p<0.05, **p<0.01, ***p<0.001 from TNBS–control, ANOVA 2-way repeated measures planned comparison). (C) The mortality rate observed in TNBS–controls (55%) was lower when treated with GB88 (8.3%) than sulfasalazine (33.3%, *p<0.05 Chi², n=11–12/group).

Figure 6. GB88 reduces macroscopic IBD-like symptoms. (A) Colonic length was reduced in TNBS–control animals (n=11) compared to sham (n=10). Both daily treatment regimes provided improvements, however, only GB88 showed statistical significance (10 mg/kg/day/p.o, n=11, sulfasalazine 100 mg/kg/day/p.o, n=11). (B) Treatment with GB88 was more effective than sulfasalazine in significantly reducing disease-induced colonic distension increases and the incidence of bowel obstruction. (C) Macroscopic tissue showed severe colonic hyperaemia, ischaemia and ulceration of TNBS–controls. (D) Severe colon adhesion to surrounding viscera was observed in TNBS–controls. GB88 significantly reduced all macroscopic pathology, whereas sulfasalazine showed a positive but non-significant effect. (E) By end point, the majority of TNBS–controls had an obstructed colon (diarrheic; score of 1. constipated/obstructed; score of 2). *p<0.05, **p<0.01, ***p<0.001 from sham, #p<0.05, ##p<0.01, ###p<0.001 from TNBS–control, ANOVA, Bonferroni planned comparison.
Figure 7. GB88 reduces colonic oedema/diapedesis in TNBS-colitis rats. (A) Colonic wet:dry ratio was significantly reduced in GB88-treated compared to TNBS−controls, which showed a large water content reflective of the oedematous state of the inflamed colon. Sulfasalazine (100 mg/kg/day p.o.) also reduced wet weight in the TNBS-affected colon, however, not to significant levels. (B) Myeloperoxidase (MPO, % change (optical density) from sham) activity was significantly increased in colonic tissue taken from TNBS-administered controls, indicative of granulocyte diapedesis. GB88 treatment (10 mg/kg/day p.o.,) ameliorated any changes in MPO, but sulfasalazine gave no significant effect. *p<0.05, **p<0.01, ***p<0.001 from sham, #p<0.05, ##p<0.01 from TNBS−control, ANOVA, Bonferroni planned comparison.

Figure 8. Representative photomicrographs of H&E stained rat colon show TNBS−control rats had severe colonic tissue damage, with extensive mucosal damage/erosion (black arrow), inflammatory cell infiltrate (white arrow), and oedema (open arrow head). Both GB88 (10 mg/kg/day) and sulfasalazine (100 mg/kg/day) caused improvement versus TNBS-controls, but GB88 prevented more of the TNBS−induced mucosal damage, inflammation and oedema than sulfasalazine.

Figure 9. GB88 improves histopathology of TNBS−induced rat colitis. Each H&E stained section was histologically scored for deterioration of colonic tissue
health in TNBS−control animals ((A) mucosa, (B) lamina propria, (C) smooth muscle and (D) bowel wall thickness)) observed in Figure 4. Both GB88 (10 mg/kg/day) and sulfasalazine (100 mg/kg/day) showed improvements in tissue health, however these were only significant for GB88 in all tissue layers. Sulfasalazine only showed improvements in smooth muscle health. ***p<0.001 from sham, #p<0.05 from TNBS−control, ANOVA, Bonferroni planned comparison.
Figure 1

% Response

Log [Antagonist]

-7 -6 -5 -4 -3 -2 -1

ENMD-1068
GB88
Figure 2
Figure 3
Figure 5
Figure 6
Figure 7
Figure 8
**Figure 9**

A. Mucosal health score

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B. Lamina propria health score

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C. Smooth muscle health score

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D. Bowel wall thickness (µm)

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*Significant difference from sham
**Significant difference from TNBS-control
###Significant difference from GB98
Significant difference from sulfasalazine
Supplemental figure 2. Cytokine expression in TNBS-treated colon homogenates measured by ELISA: (A) TNFα and (B) IL-6. Tissue health affected the cytokine expression, with necrotic tissue giving unexpected low cytokine expression in diseased (control) tissue compared to GB88 or sulfasalazine treated tissue. No differences in cytokine expression was observed.

Antagonism of protease activated receptor 2 protects against experimental colitis Rink-Jan Lohman, Adam J. Cotterell, Jacky Suen, Ligong Liu, Tram Anh Do, David Vesey and David P. Fairlie. JPET.
Supplemental figure 1. (A) Photomicrographs of H&E stained rat colon sections treated in vivo with either an intracolonic dose of saline, SLIGRL-NH2 (1 mg/rat) or SLIGRL-NH2 (1 mg/rat) and oral GB88 (10 mg/kg p.o). When given two hours before, GB88 prevented the oedema induced by intracolonic SLIGRL-NH2. Note that the submucosa appears thicker in SLIGRL-NH2 only treated colons, and there is a distinct lack of overt inflammatory cell invasion to any lamina of the colon (arrows). (B) This lack of cell invasion is also highlighted in the MPO assay performed on the same colons, which clearly shows a lack of change in MPO activity between saline, SLIGRL-NH2 or SLIGRL-NH2 and GB88-treated, as also shown in wet dry ratio (see main text). These data suggest that SLIGRL-NH2 at this dose may not cause diapedesis of inflammatory cells, but transport of water only.

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### Score Criteria

**Ulceration**

- 0. No Damage
- 1. Focal hyperaemia
- 2. Ulceration without hyperaemia or bowel wall thickening
- 3. Ulceration with inflammation at 1 site
- 4. ≥2 sites of ulceration and inflammation
- 5. Major sites of inflammation ≥1(<2)cm along bowel
- 6-10 Major sites of inflammation ≥2cm along bowel (gradiented with affected length).

**Adhesions**

- 0. No adhesion
- 1. Minor adhesion
- 2. Major adhesion

**Faecal consistency**

- 0. Normal pellets
- 1. Diarrhoea
- 2. Constipation/bowel obstruction (includes rupture)

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**Supplementary Table 1:** Criteria for scoring macroscopic damage to TNBS- colitis in rats. Derived and amended from (Bobin-Dubigeon et al., 2001)

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SUPPLEMENTAL INFORMATION

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JPET

Supplemental Methods

Disease activity index (DAI).

DAI for both acute and chronic models was assessed and scored by an expert blinded to treatments, using the following criteria: Mobility: 0; normal movement. 1; sluggish movement/abnormal gait. 2; reduced mobility/inactivity. 3; complete immobility/lack of posture and righting reflex. Gastro-intestinal pathology: 0: normal. 1; loose stools/soft faeces. 2; mild diarrhoea mild haemocult. 3; severe diarrhoea, gross rectal bleeding, palpable mass in abdomen (‘toxic megacolon’/bowel obstruction). Discomfort response to abdominal palpitation: 0; no abnormal responses. 1; mild vocalisation. 2; increased vocalisation with flinching during handling. 3; excessive spontaneous vocalisation. Sickness behaviours: 0; none. 1; ruffled fur, reduced grooming. 2; hunched abdominal and sunken facial appearance, ruffled fur and lack of grooming. 3; severe chromodacryorrhoea, excessively soiled rear, no grooming, hunched appearance. Any animal that reached stage 3 in any criteria was euthanised by CO₂ inhalation, as stipulated in the ethical agreement, and recorded as a
disease-related mortality. Scores were summed and expressed as total disease score (maximal possible score =12). DAI was measured at end point only (10h) in the acute study, and daily in the chronic TNBS study.

**Macroscopic disease index.**

Colons were dissected postmortem and given a macroscopic disease score based on that previously described (Bobin-Dubigeon et al., 2001; Woodruff et al., 2005) with some minor alterations (Supplementary Table 1). Briefly, dissected colons were scored for ulceration, adhesions to surrounding viscera and stool consistency. Bowel obstruction was given a score of 2 in the latter criteria. The maximal score possible was 14. The entire colon length was removed between the ileo-colic and colo-rectal junction. Length and maximal distension width were measured. Sections of affected colon were taken for biochemical analysis, histology and wet:dry weight.

**Histology and immunohistochemistry.**

Tissue samples were embedded in paraffin wax, cut on a microtome (5 μm) and stained with either Haematoxylin and Eosin (H&E), or alcian Blue (pH 1.0) and safranin–O. H&E–stained colon sections were examined by an expert examiner blinded to treatment conditions for inflammation and IBD–like histopathologies and scored appropriately for severity, using a modified system.(Lubbad et al., 2009) Each layer of tissue (Mucosa, submucoasa and smooth mucles layers) was independently scored: 0; normal tissue. 1; mild damage/mild diapedesis. 2; moderate architectural damage, small ulcers forming, submucosal diapedesis. 3; severe tissue structure damage, pseudo-membrane crypt formation, mucosal
loss, extensive ulceration and/or necrosis, transmural diapedesis. Scores were expressed as a total sum (maximum 9).

Differential alcian blue/safranin-O stained sections were used for mast cell and mucin depleted cell counts, based on glycosaminoglycan (Safranin-O) or mucin (alcian blue) content of the cell. (Lam and Schmidt, 2010) Briefly, mast cells stained red were considered inactive, those blue degranulated, and those with both red and blue staining were considered active. Three sections per rat were used for cell counts. Mucosal goblet cells stained positive for alcian blue were counted as either blue, purple or red stained from five images of random mucosal regions (photographed at 40x lens). Data was expressed as a total percent of goblet cells. Blue or purple represents stained high and medium charged carbohydrate mucin, respectively (Linden et al., 2008). Red cells (safranin-O–stained only) were assumed void of mucin.

Immunohistochemistry was performed using antibodies raised against PAR2 (Primary; N19 1:100, Santa Cruz. Secondary; Gt–anti–Rb Alexa 488, 1:200 Invitrogen) and double label experiments used PAR2 (Primary; N19 1:100, Santa Cruz. Secondary; Gt–anti–Rb Alexa 546, 1:200 Invitrogen) and mast cell tryptase (AA1, 1:100, Abcam, secondary, Rb-anti-Ms Alexa 488, 1:200, Invitrogen) using standard 10mM citrate antigen retrieval and 4h incubation protocols. Sections stained with anti-PAR2 antibody were analysed for positively stained cells with either a predominant membrane (annular-like) or intracellular (somatic-like) staining pattern, similar to that previously described (Amadesi et al., 2006), and expressed as a percentage of total positive cells counted. All fluorescence
images were subject to background subtraction and brightness enhancement using standard ImageJ algorithms to clarify staining patterns. Fluorescence intensity was not measured.


