Antagonism of Protease-Activated Receptor 2 Protects against Experimental Colitis

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ABSTRACT

Many trypsin-like serine proteases such as 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 probably 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 (5-isoxazoyl-Cha-Ile-spiro[indene-1,4′-piperidine]; GB88) was evaluated for the blockade of intracellular calcium release in colonocytes and anti-inflammatory activity in acute (PAR2 agonist-induced) versus chronic [2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced] models of colitis in Wistar rats. Disease progression (disease activity index, weight loss, and mortality) and postmortem colonic histopathology (inflammation, bowel wall thickness, and myeloperoxidase) were measured. PAR2 and tryptase colocalization were investigated by using immunohistochemistry. GB88 was a more potent antagonist of PAR2 activation in colonocytes than another reported compound, [N′-3-methylbutyryl-N′′-6-aminohexanoyl-piperazine (ENMD-1068) (IC50 8 μM versus 5 mM). Acute colonic inflammation induced in rats by the PAR2 agonist SLIGRL-NH2 was inhibited by oral administration of GB88 (10 mg/kg) with markedly reduced edema, mucin depletion, PAR2 receptor internalization, and mastocytosis. Chronic TNBS-induced colitis in rats was ameliorated by GB88 (10 mg/kg/day p.o.), which reduced mortality and pathology (including colon obstruction, ulceration, wall thickness, and 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 after stenosis-related bowel perforation (Andersson and Söderholm, 2009), colorectal carcinoma (Laukootter et al., 2011), and multiple organ dysfunction. Tissue biopsies and colonic luminal fluid from human patients with IBD and 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 because of their pleiotropic functions in vivo. Clinical and experimental studies have shown broad-spectrum and more specific protease inhibitors, such as nafamostat mesilate, have been used with some efficacy in relieving colitis-like symptoms (Isozaki et al., 2006; Lee et al., 2010); however, their use may be limited

ABBREVIATIONS: CD, Crohn’s disease; UC, ulcerative colitis; IBD, inflammatory bowel disease; PAR2, protease-activated receptor 2; TNBS, 2,4,6-trinitrobenzenesulfonic acid; ANOVA, analysis of variance; ENMD-1068, N′-3-methylbutyryl-N′′-6-aminohexanoyl-piperazine; MPO, myeloperoxidase; DAI, disease activity index; H&E, hematoxylin and eosin; ELISA, enzyme-linked immunosorbent assay; GB88, 5-isoxazoyl-Cha-Ile-spiro[indene-1,4′-piperidine].
because of the likely involvement of multiple proteases in IBDs. Serine proteases (e.g., trypsin and β-trypsinase) 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 nonproteolytic 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). The precise roles for protease-mediated PAR2 signaling in the gut are not defined, but PAR2 and its activating proteases are important in the gastrointestinal tract (Kawabata et al., 2008; Browning, 2010), and there is evidence of a pathogenic role for PAR2 in CD and UC (He et al., 2004; Hansen et al., 2005; Browning, 2010) and irritable bowel syndrome (He, 2004; Barbara and Cremon, 2008; Lee et al., 2010). PAR2 is up-regulated on various cell types in colonic tissue taken from patients with IBD and irritable bowel syndrome (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) and chloride-mediated increases in colonic permeability and edema (Cenac et al., 2002), leading to diapedesis and bacterial translocation across the protective mucosa (Róka et al., 2007; Lee et al., 2010). Furthermore, intracellular 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 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced (Hyun et al., 2008) or bacteria-induced (Hansen et al., 2005; Cottrell et al., 2007) colitis. An efficacious PAR2 antagonist could help to clarify in vivo roles for PAR2 and its activating proteases in the etiology 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 nonpeptide agonists. Here, we evaluate the efficacy of one such antagonist [5-isoxazoyl-Cha-Ile-spiro[indene-1,4′-piperidine] (GB88); 10 mg/kg/day p.o.] in rat models of acute and chronic inflammatory bowel disease induced by a PAR2 agonist (SLGRL-NH2) or TNBS, respectively. GB88 was effective in ameliorating IBD-like symptoms in both rat models, being 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 verify PAR2 antagonism as an effective strategy for treating IBDs.

## Materials and Methods

**Animals.** Male Wistar rats (aged 8–9 weeks, 250–300 g), bred and housed at the Australian Institute for Bioengineering and Nanotechnology (Brisbane, Australia), 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 (Queensland, Australia).

**Drugs, Peptides, and Chemicals.** Pircylsulfonic acid (TNBS) and the nonsteroidal anti-inflammatory drug sulfasalazine were supplied by Sigma-Aldrich (Sydney, Australia). PAR2-activating peptides SLGRL-NH2 and 2Furolyl-LIGRLO-NH2 were synthesized by standard solid-phase peptide chemistry. PAR2 antagonists GB88 (Suen et al., 2011) and N′-3-methylbutyryl-N′-6-aminohexanoyl-piperazine (ENMD-1068) (Kelso et al., 2006) were synthesized 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 characterized by proton nuclear magnetic resonance spectroscopy. GB88 and sulfasalazine were dissolved in olive oil (generic brand) and administered by oral gavage (polypropylene feeding tubes, 18 G × 75 mm; Instech Soloman; Walker Scientific, Wangara, Perth, Australia; ≈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 before the addition of 2Furolyl-LIGRLO-NH2 (1 µM). Intracellular calcium mobilization was measured by differences in fluorescence plotted against corresponding antagonist concentrations, enabling the 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 and anesthetized with isoflurane for 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 GB88 and sulfasalazine were dissolved in olive oil (generic brand) and administered by oral gavage (polypropylene feeding tubes, 18 G × 75 mm; Instech Soloman; Walker Scientific, Wangara, Perth, Australia; ≈500 µl).

**Macroscopic Disease Index.** Colons were dissected postmortem and given a macroscopic disease score based on that described previously (Bobin-Dubigeon et al., 2001) with some minor alterations (maximal score 14; see Supplemental Table 1). The entire colon length was removed between the ileocolic and colorectal 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 hematoxylin 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 Biotechnology, Inc., Santa Cruz, CA) and/or mast cell tryptase (AA1; 1:100; Abcam Inc., Cambridge, MA) by using standard 10 mM citrate antigen retrieval and 4-h (primary) incubation protocols. All fluorescence images were subject to background subtraction and brightness enhancement by using standard ImageJ algorithms (National Institutes of Health, Bethesda, MD) 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 the distance from outer circular muscle to the inner crypt base was measured.

**Myeloperoxidase and ELISA.** Unfixed colon sections were homogenized in 4°C/0.5% hexadecyl trimethylammonium bromide/phosphate-buffered saline buffer (100 mg/ml w/v, pH 6.0) and centrifuged at 13,000 rpm for 10 min at 4°C. Supernatant (100 μl) was transferred (in duplicate) to a 96-well plate to which o-dianisidine (20 μl, 2.85 mg/ml in 1% H2O2/phosphate-buffered saline) was added and mixed by aspiration. The plate was allowed to incubate for 15 min at room temperature (in the dark) then transferred to a fluorimeter (FLUOstar Optima; BMG Labtech GmbH, Offenburg, Germany). Absorbance was read at λ = 450 nm. Data were expressed as absolute optical density units. Cytokine expression (tumor necrosis factor α and interleukin-6) in tissue homogenates was measured by using ELISA (BD Biosciences, San Jose, CA) according to the manufacturer’s instructions.

**Data Analysis.** Experimental results were expressed as mean ± S.E.M. Data were analyzed by using Prism software (v5.0a; GraphPad Software, San Diego, CA). Statistical comparisons were performed by using two-way repeated-measures ANOVA for temporal data sets involving three or more groups. For individual time points, data were analyzed with one-way ANOVA, and groups were compared by using Bonferroni planned comparisons. Significance was set at p < 0.05; Fig. 2, A 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-NH2-induced wall thickness changes, histopathology, and edema (wet/dry weight; p > 0.05; Fig. 2, A–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). Likewise, no changes in MPO expression in tissue homogenates were observed (Supplemental Fig. 1). These data confirm that PAR2 activation promotes colonic edema with little leukocyte diapedesis (Cenac et al., 2002).

**Results**

**GB88 Is a PAR2 Antagonist in Colon Cells.** GB88 was a potent antagonist of intracellular Ca2+ release induced in HT29 adenocarcinoma cells by the PAR2 agonist 2-Furoyl-LIGRLO-NH2 (IC50 8 μM; Fig. 1) (Suen et al., 2011). This in vitro antagonist potency is similar to what we found in other cell types (IC50 2–5 μM), when we also showed that GB88 was specific for PAR2 over PAR1 in vitro and in a rat paw edema assay (Suen et al., 2011). Moreover, here we show that GB88 was also three orders of magnitude more potent than ENMD-1068 (IC50 5 mM; Fig. 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-NH2 (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 DAI (diarrhea, chromodacryorrhea, reduced grooming, and mobility) compared with sham (n = 5) as reported previously (Cenac et al., 2002). Postmortem analysis confirmed that SLIGRL-NH2-induced mild colonic hyperemia and bowel wall thickening caused by edema. Microscopically, colons showed distinct increases in wall thickness and histopathological scores (p > 0.05; Fig. 2, A and B, respectively). Wet/dry weight ratio was also significantly increased (p > 0.05; Fig. 2C). 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 (Lindén et al., 2008). The PAR2 antagonist GB88 prevented these changes in mucin expression (Fig. 3, A, top, and B).

PAR2 immunofluorescence was located in colonic mucosa, particularly lining the epithelium, but also in the membrane and soma of goblet cells (Fig. 3A, bottom). The expression of PAR2 became more intense in the soma of goblet cells and less on the membrane at 10 h after SLIGRL-NH2 treatment, suggesting the activation of PAR2 causes its internalization in goblet cells (Fig. 3, A, bottom, and 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 internalized in goblet cells in which mucin levels are depleted. Such effects on mucin may contribute to disease (Lindén et al., 2008).

Tryptase-positive cells (probably mast cells) were found in the lamina propria and mucosal crypts. There was also a pericellular staining pattern in the mucosal crypts, which

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**Fig. 1.** GB88 is a potent antagonist of Ca2+ mobilization in HT29 colonocytes. Intracellular Ca2+ release in HT29 colonocytes induced by 2F-LIGRLO-NH2 (1.0 μM) in the presence of varying concentrations of GB88 is shown. GB88 was three orders of magnitude more potent as a PAR2 antagonist than as reported for compound ENMD-1068.
probably represents extracellular tryptase surrounding goblet cells, because it colocalized with PAR2 immunoreactivity (Fig. 4). Because of the paucity of staining, tryptase levels could not be accurately analyzed between treatment groups. There was, however, little evidence to suggest that tryptase-positive colonic mast cells had any recognizable PAR2 immunoreactivity (Fig. 4).

**TNBS-Induced Colonic Disease Progression.** GB88 was further examined in a more nonspecific 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 diarrhea, but recovered by day 4. These symptoms probably result from the ethanol vehicle routinely used in the induction phase in this assay. TNBS-treated control animals had a DAI that progressively deteriorated (Fig. 5A), which evidently was terminal (Fig. 5C). Intestinal symptoms in this group consisted of a palpable abdominal mass, which was evident in 7 of 11 control animals. In all groups body weight deteriorated until day 3, after which weight began to increase, returning to baseline levels in all but TNBS-control animals (Fig. 5B). Such weight regain is artifactual because of 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.

**Fig. 2.** PAR2-induced colonic inflammation. A to C, intracolonic administration of SLIGRL-NH₂ (1 mg/rat) induced an increased bowel wall thickness (*, p < 0.05 from sham; n = 6; ANOVA planned comparison) (A), a mild but significant colonic inflammation (**, p < 0.01 from sham; n = 5) (B), and an increased wet/dry ratio (*, p < 0.05 from sham) (C). Pretreatment 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-NH₂ (*, 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-NH₂, as determined by Alcian Blue/Safranin-O staining, but this finding was not statistically significant (p = 0.06).
onward (Fig. 5A), compared with TNBS-controls, exceeding baseline weights at endpoint. 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 versus 33.3% mortality, respectively, p < 0.05; Fig. 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. 6, A–D). Both treatments were effective at reducing incidence of macroscopic disease symptoms, particularly bowel obstruction (p < 0.05; Fig. 6E).

**Edema.** Colons taken from TNBS-controls had significantly greater wet/dry ratios than shams, confirming edema (p < 0.05; Fig. 7A). Even though they did not return 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 (hemorrhage, transmural ischemia, and necrosis) (Fig. 8), symptoms synonymous with human (nonspecific) 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, edema, and diapedesis in all tissue layers compared with TNBS-controls (Fig. 9, A–C, respectively). Bowel walls were thickest in TNBS-controls, which were normalized to sham levels by GB88 \( (p < 0.05; \text{Fig. } 9D) \). 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 edema and diapedesis. All TNBS-treated colons were sectioned and stained for mast cells and mucin by using Alcian Blue/safranin-O; however, because of the advanced necrotic state of colic tissue taken from TNBS-control animals viable cells could not be accurately counted in these tissues. The advanced necrotic state of the tissue also influenced cytokine expression, because TNBS-control colon homogenates had unexpectedly low lev-

**Fig. 4.** PAR2 and tryptase colocalize in colonic mucosa. A, PAR2 immunoreactivity (red) shows PAR2 is distributed throughout goblet cells. Tryptase (green) expression seems 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, the absence of primary antibodies in negative controls shows no specific immunoreactivity.

**Fig. 5.** GB88 reduces TNBS-induced disease-like symptoms. A and B, 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 DAI (*, \( p < 0.05 \); **, \( p < 0.01 \); ***, \( p < 0.001 \) from sham; \( n = 10 \); ANOVA two-way repeated-measures planned comparison) (A) and weight regain as observed in TNBS-control rats (***, \( p < 0.001 \) from TNBS-control; ANOVA two-way repeated-measures planned comparison) (B). C, the mortality rate observed in TNBS-controls (55%) was lower when treated with GB88 (8.3%) than sulfasalazine (33.3%; *, \( p < 0.05 \); **, \( p < 0.001 \) from TNBS-control; \( n = 11–12/group \).
GB88 reduces macroscopic IBD-like symptoms. A, colonic length was reduced in TNBS-control animals (n = 11) compared with sham (n = 10). Both daily treatment regimes provided improvements; however, only GB88 (10 mg/kg/day p.o.; n = 11) showed statistical significance (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, ischemia, 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 nonsignificant effect. E, by endpoint, 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.

GB88 reduces colonic edema/diapedesis in TNBS-colitis rats. A, colonic wet/dry ratio was significantly reduced in GB88-treated compared with TNBS-controls, which showed a large water content reflective of the edematous 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, MPO (percentage 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 had 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.
els of cytokines (tumor necrosis factor α and interleukin-6) in ELISA compared with 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 (edema, mastocytosis, mucin depletion, and 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 internalization 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 (Lindén 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, etc.).

![Fig. 8. Representative photomicrographs of H&E-stained rat colon show TNBS-control rats had severe colonic tissue damage with extensive mucosal damage/erosion (black arrows), inflammatory cell infiltrate (white arrows), and edema (open arrowhead). 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 edema than sulfasalazine.](image)

![Fig. 9. GB88 improves histopathology of TNBS-induced rat colitis. Each H&E-stained section was histologically scored for deterioration of colonic tissue health in the TNBS-control animals (mucosa (A), lamina propria (B), smooth muscle (C), and bowel wall thickness (D)) observed in Fig. 4. Both GB88 (10 mg/kg/day) and sulfasalazine (100 mg/kg/day) showed improvements in tissue health; however, these were significant only for GB88 in all tissue layers. Sulfasalazine showed improvements only in smooth muscle health. *, p < 0.05 from sham; **, p < 0.001 from sham; #, p < 0.05 from TNBS-control, ANOVA, Bonferroni planned comparison.](image)
and Clostridium difficile) (Hansen et al., 2005; Cottrell et al., 2007), and allergen-derived proteases (cockroaches and dust mites) (Shpacovitch et al., 2008; Day et al., 2010). Although concentrations of PAR2-activating serine proteases (i.e., trypsin and β-trypsin) 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 is responsible for zonula occludens-1-related tight junction reorganization, 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-trypsin (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). Because PAR2 is responsible for a mild form of colonic inflammation, it seems likely that degranulating mast cells produce β-trypsin (Hamilton et al., 2011) that causes the PAR2 mediated colitis symptoms (hyperemia, edema, mastocytosis, and mucin depletion) observed here. This is supported by our observations of colocalization of PAR2 and trypsin expression in and around goblet cells, the induced mucin depletion within these cells, and the blockade of inflammation, mucin depletion, and mastocytosis by the PAR2 antagonist GB88.

Various serine proteases and their precursors [e.g., trypsinogen (Hansen et al., 2005; Cottrell et al., 2007) and trypsines (He and Xie, 2004; He et al., 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), they probably also disrupt the 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.

Because we found little evidence of PAR2 expressed on β-trypsin-containing mast cells in our immunohistochemistry study, perhaps the reported mast cell degranulation is not directly caused by 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, neuropetides (i.e., substance P and vasoactive intestinal peptide) released from PAR2-activated c-fibers 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 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-NH$_2$- and TNBS-induced experimental colitis in rats. Our results link the pathogenesis of PAR2 agonist-induced colonic inflammation with mastocyto- sis and coexpression of β-trypsin and PAR2 around mucin-depleted goblet cells. It is noteworthy that β-trypsin has recently been linked to colitis through a gene deletion study (Hamilton et al., 2011). The current results connect PAR2 activation to β-trypsin 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.

Authorship 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.

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