Newly developed serine protease inhibitors decrease visceral hypersensitivity in a post-inflammatory rat model for irritable bowel syndrome

NEWLY DEVELOPED SERINE PROTEASE INHIBITORS DECREASE VISCERAL HYPERSENSITIVITY IN A POST-INFLAMMATORY RAT MODEL FOR IRRITABLE BOWEL SYNDROME.

Running title: Serine proteases in visceral hypersensitivity

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ABSTRACT

Background and purpose

Serine proteases are suggested as important players in visceral pain. We investigated their effect by using newly developed serine protease inhibitors with a well-characterized inhibitory profile in a post-inflammatory IBS rat model.

Experimental Approach

Colitis rats received intrarectal TNBS and controls 0.9% NaCl. Colonoscopies were performed on day 3, to confirm colitis, and later on until mucosal healing. Visceral hypersensitivity was quantified by visceromotor responses (VMR) to colorectal distension, 30 min after intraperitoneal injection of the serine protease inhibitors nafamostat, UAMC-00050 or UAMC-01162. Serine proteases, protease-activated receptors (PARs) and transient receptor potential channels (TRPs) were quantified via qPCR and immunohistochemistry. Proteolytic activity was characterized using fluorogenic substrates.

Key results

VMR was significantly elevated in post-colitis rats. Nafamostat normalized VMRs at the lowest dose tested. UAMC-00050 and UAMC-01162 significantly decreased VMR dose-dependently. mRNA expression of tryptase-αβ-1, PAR4 and tryptase immunoreactivity were significantly increased in the colon of post-colitis animals. Accordingly, trypsin-like activity was significantly increased in the colon but not in the feces. PAR2 and TRPA1 immunoreactivity colocalized with CGRP-positive nerve fibers in control and post-colitis animals.

Conclusions and implications

The increase in serine protease expression and activity together with the increased expression of downstream molecules at the colonic and DRG level and in CGRP positive sensory nerve fibers point to a role for serine proteases in post-inflammatory visceral hypersensitivity. The results of this study call for further investigation of serine protease inhibitors as an interesting treatment strategy for IBS related visceral pain.

KEYWORDS

Visceral hypersensitivity; Irritable bowel syndrome; serine proteases.

ABBREVIATIONS

AP activating peptide

ATP adenosine triphosphate

CGRP calcitonin gene-related peptide

DRG dorsal root ganglia

EMG electromyographic electrodes

GEE generalized estimating equations

GPCR G protein-coupled receptor

IBS irritable bowel syndrome

IBS-C irritable bowel syndrome constipation

IBS-D irritable bowel syndrome diarrhea

IC_{50} half maximal inhibitory concentration

i.p. intraperitoneal

KLK kallikrein

LSD least significant difference

MPO myeloperoxidase

PAR protease-activated receptor

PBS phosphate-buffered saline

PRSS protease serine, trypsin precursor

qPCR quantitative polymerase chain reaction

SEM standard error of the mean

S-N-K student-newman-keuls

TNBS trinitrobenzenesulphonic acid

TRPA transient receptor potential ankyrin channel

TRPV transient receptor potential vanilloid channel

uPA urokinase plasminogen activator

VMR visceromotor response

INTRODUCTION

Irritable bowel syndrome (IBS) is a functional gastrointestinal disorder, characterized by chronic abdominal pain and altered defecation patterns, in the absence of any organic cause(Chey et al., 2015). Visceral hypersensitivity is a main factor underlying abdominal pain in IBS patients(Barbara et al., 2011) and an important contributor to gastrointestinal symptom generation in IBS patients, thus making it a relevant treatment target(Simren et al., 2017). Remarkably, only 24% of the IBS patients report complete relief of abdominal pain after treatment, which is usually only a symptomatic treatment of the most explicit motility-related symptom(Hungin et al., 2003). Therefore, further research towards new treatments for abdominal pain in IBS patients is of utmost importance.

Gastrointestinal inflammation is considered as an important trigger for the onset of visceral hypersensitivity(De Schepper et al., 2008). Upon activation of inflammatory cells (e.g. mast cells, T-/B-cells) at the peripheral level, excessive amounts of mediators (e.g. histamine, serotonin, cytokines, proteases, bradykinin, ATP) are released, thereby sensitizing peripheral afferent neurons contributing to visceral hypersensitivity. Considerable research efforts are devoted to the role of various inflammatory mediators in visceral hypersensitivity but the role of proteases only starts to be understood(Ceuleers et al., 2016; Vergnolle, 2016).

Especially the class of serine proteases attracts attention in visceral nociception research because elevated serine protease expression and/or activity is repeatedly found in colonic and fecal samples of IBS patients(Annahazi et al., 2009; Barbara et al., 2004; Buhner et al., 2009; Cenac et al., 2007; Gecse et al., 2008; Roka et al., 2007; Tooth et al., 2014). Despite suggestions of serine protease inhibitors as a possible treatment option for visceral pain, only few preclinical animal studies actually investigated the effect of serine protease inhibitors on visceral sensitivity(Ceuleers et al., 2016; Vergnolle, 2016). A clear role for protease-activated receptors (PAR) in visceral hypersensitivity is shown in preclinical *in vitro* and *in vivo* studies but only few animal studies have been conducted evaluating serine protease inhibitors, thereby focusing on proteases rather than PARs. It is shown

that supernatant from colonic biopsies from IBS patients loses its capacity to induce visceral hypersensitivity in mice if the supernatant is pretreated *in vitro* with nafamostat, a broad-spectrum serine protease inhibitor(Cenac et al., 2007; Wang et al., 2015). However, proof of an *in vivo* effect remains scarce. To our knowledge, the only evidence that *in vivo* serine protease inhibition reduces visceral pain was provided by Zhao et al., showing that intragastric pre-treatment with camostat, structurally related to nafamostat, reduced pain in a rat model of stress-induced visceral hypersensitivity(Zhao et al., 2011). This highlights the importance of more extensive research on serine protease inhibitors as a possible treatment option for visceral pain.

Therefore, we aimed at investigating the *in vivo* symptomatic effect of two newly developed serine protease inhibitors (UAMC-00050 and UAMC-01162; patent WO2007045496(Joossens et al., 2007)) in a post-inflammatory rat model for IBS. We compared the effects of UAMC-00050 and UAMC-01162 to those of nafamostat and attempted to unravel the type of serine proteases contributing to visceral hypersensitivity to define the optimal inhibition profile for serine protease inhibitors targeting abdominal pain in IBS patients.

MATERIAL & METHODS

Animals

Male Sprague-Dawley rats (200-225g; Charles River, Italy) were housed at constant room temperature (22 ± 2°C) and humidity (60%) with two rats per cage. Rats had unlimited access to water and food and were kept on a 12h:12h day-night cycle. All experiments were approved by the Ethical Committee for Animal Experiments of the University of Antwerp (EC nr. 2014-41) and performed in accordance with Directive 2010/63/EU of the European Parliament and the Council on the protection of animals used for scientific purposes.

Induction of TNBS-colitis

2,4,6-trinitrobenzenesulphonic acid (TNBS) colitis was induced at day 0 by a TNBS-enema containing 4 mg TNBS (Sigma-Aldrich Inc, USA) in 50% ethanol (Acros Organics, Germany) as previously described(Deiteren et al., 2015). This post-TNBS rat model for IBS is validated and routinely used in our lab and described in review papers as a relevant animal model to study visceral pain(Deiteren et al., 2015; Greenwood-Van Meerveld et al., 2015). After an overnight fast and under ketamine (35 mg/kg i.p.; Ketalar® Pfizer) and xylazine (5 mg/kg i.p.; Rompun® Bayer) anesthesia, 0.25 ml of the TNBS solution was administered intrarectally using a flexible catheter (18G, length 4.5 cm). Control animals received 0.25 ml 0.9% NaCl (Braun®, Belgium) intrarectally, under ketamine (35 mg/kg i.p.) and xylazine (5 mg/kg i.p.) anesthesia. The animals were kept in tail-up position during 1 min and were then allowed to recover in a Trendelenburg position in a temperature-controlled cage (28°C) up until 1h until they regained consciousness. No post-operative analgesia was administered as approved by the Ethical Committee for Animal Experiments of the University of Antwerp (EC nr. 2014-41). Subsequently, animals were brought back to their cages with free access to food and water.

Experimental design

The experimental course is shown in figure 1. A mild colitis was induced on day 0 by a TNBS-enema. Controls received an intrarectal administration with 0.9% NaCl. Colitis was verified by colonoscopy on day 3. From day 10 onwards, colonoscopy was performed every 4 days to follow up the healing of the colonic mucosa. Functional experiments (VMR) were performed three days after complete resolution of colitis. The compounds (0.1-10 mg/kg nafamostat mesilate, 0.01-1 mg/kg UAMC-00050, 1-2.5 mg/kg UAMC-01162) or vehicle (sterile water for nafamostat, 5% DMSO for UAMC-00050 and UAMC-01162) were injected intraperitoneally (i.p.) 30 min before the start of the VMR experiment (n=7-10/group). Group sizes are unequal due to experimental loss (electrode failure, anesthesia, reamining colonic inflammation). The active compounds are described in detail in the materials sub-section. Colonic compliance was evaluated before animals were sacrificed (exsanguination under 45 mg/kg i.p pentobarbital anesthesia) to asses colonic inflammatory parameters (colonoscopy, macroscopy, microscopy and myeloperoxidase activity (MPO)). Colonic samples were taken for immunohistochemistry, qPCR and proteolytic activity experiments. DRG samples were obtained from another group of control and post-colitis IBS rats (n=12/group). All animals were randomised for treatment and data analysis was carried out by a person blinded to this study.

Visceromotor response

The visceromotor response (VMR) is the nociceptive reflex in which abdominal muscles contract in response to a colorectal balloon distension(Ness & Gebhart, 1988). This is a validated and objective method to quantify visceral sensitivity in rats(Deiteren et al., 2014; Deiteren et al., 2015; Vermeulen et al., 2013). Under ketamine (35 mg/kg i.p.) and xylazine (5 mg/kg i.p) anesthesia, non-isolated ends of a pair of electromyographic (EMG) electrodes were implanted into the external abdominal muscle and the other electrode ends were subcutaneously tunneled to the neck, exteriorized and accessibly immobilized in between the shoulder blades. Rats were allowed to recover in a temperature-

controlled cage (28°C) up until 1h until they regained consciousness. No post-operative analgesia was administered as approved by the Ethical Committee for Animal Experiments of the University of Antwerp (EC nr. 2014-41). Subsequently, animals were brought back to their cages with free access to food and water. Three days later, the VMR was quantified in conscious rats by introducing into the colorectum a lubricated balloon (length 5 cm) which was connected to a barostat (Distender Series II Barostat, G&J Electronics, Canada). The EMG signal was recorded by a data-acquisition during a phasic distension protocol (10-20-30-40-60 mmHg, 20 s, 4 min interval) generated by the barostat. The EMG signal was registered, amplified (Neurolog, Digitimer Ltd, UK), digitalized (CED 1401, Cambridge Electronic Design, UK) and analyzed using Spike2 V.5.16 (Cambridge Electronic Design, UK). The VMR was quantified by calculating the area under the curve (AUC) of the EMG signal during colorectal distension (20 s) corrected for the EMG signal before the distension (20 s).

Colonic compliance

Colonic compliance is the resistance of the colon against deformation and calculated as the ratio of change in volume over change in pressure. The effect of the serine protease inhibitors was studied on colonic compliance to assess possible changes in the viscoelastic properties of the colon. Under pentobarbital anesthesia (45 mg/kg, i.p. Nembutal®), a lubricated balloon was introduced into the colorectum of the rat and filled with increasing volumes of water (0-0.5-1.0-1.5-2.0 ml, 80s interval). The corresponding pressure in the colon of the rat was measured and the resulting pressure-volume curves display colonic compliance.

Inflammatory parameters

Colonoscopy

Colonoscopy was performed with a pediatric endoscope (Olympus GIF-N30, Olympus Europe GmbH). Under ketamine/xylazine anesthesia (35/5 mg/kg, i.p.), the lubricated tip of the endoscope was introduced into the colon and advanced under endoscopic view until the hepatic flexure was

reached (± 10 cm proximal to the anus). During withdrawal of the endoscope, intestinal inflammation was evaluated using a standardized scoring system (total score 0-19)(Vermeulen et al., 2011).

Post-mortem inflammatory markers

At the end of the experiments, the rat was sacrificed and the colon was isolated, rinsed with Krebs solution, opened longitudinally and macroscopically evaluated using a validated scoring system (total score 0-10) (Vermeulen et al., 2011).

For microscopic evaluation, a colonic segment of approximately 1 cm^2 was fixed in 4% formaldehyde, embedded in paraffin and stained with hematoxylin & eosin. A microscopic score of 0-10 was given using a previously published scoring system(Vermeulen et al., 2011).

Finally, colonic myeloperoxidase (MPO) activity was measured as previously published(Vermeulen et al., 2011). The MPO activity is defined as the quantity needed to transform 1 µmol H_2O_2 to H_2O within 1min at 25°C and is expressed as unit/g tissue(Pulli et al., 2013).

Quantitative RT-PCR

Rats were sacrificed and distal colonic segments of approximately 50 mg were collected. Dorsal root ganglia (DRGs) T13-L2 (splanchnic colonic afferents) and L6-S1 (pelvic colonic afferents) were harvested bilaterally. Briefly, the spinal column was dissected, cut down the mid-line and after removal of the spinal cord and meninges, DRGs of interest were extracted as previously described(Sleigh et al., 2016). Samples were snap-frozen in liquid nitrogen and stored at -80°C. Total RNA was extracted from colon or DRG (Isolate II RNA Mini Kit, Bioline) and RNA was converted to cDNA by reverse transcription (SensiFAST cDNA Synthesis Kit, Bioline). A Taqman gene expression assay (Thermofisher, list of primers in appendix 1) was executed on an ABIPrism 7300 sequent detector system (Applied Biosystems, USA) in a 25µl reaction volume containing 2µl cDNA, 12.5µl Taqman Universal PCR Master Mix (ThermoFisher), 1.25µl Taqman assay probe and 9.25µl RNase-

free H_2O. Gene expression assays were executed following the MIQE guidelines(Bustin et al., 2009). GAPDH and β-actin were reference genes. The parameters for PCR amplification were 50°C for 2min, 95°C for 10min followed by 40 cycles of 95°C for 15s and 60°C for 1min(Bustin et al., 2009). The outcome values were analyzed using qBASE^{PLUS} software (Biogazelle N.V., Zwijnaarde, Belgium).

Immunohistochemistry for mast cell tryptase

Colonic samples were fixed (4% formaldehyde; Merck, Germany), embedded in paraffin and cut into 5 µm sections. Sections were pretreated with trypsin (37°C, 10 min; Sigma Aldrich, USA) and citrate buffer pH6 (microwave, 10 min). Slides were overnight incubated in a moist chamber with mouse mast cell tryptase monoclonal antibody (1:10.000; clone AA1; Abcam, UK). After Tris-saline buffer wash, slides were incubated (30 min) with biotinylated goat anti-mouse IgG antibody (1:200; Vector Laboratories, USA) and rat serum (1:20; Vector Laboratories, USA) and incubated (60 min) with a Vectastain® avidin-biotin complex (Vector Laboratories, USA). Slides were washed, developed in an aminoethylcarbozole solution with hydrogen peroxide (Sigma Aldrich, USA) (10 min), counterstained with hematoxylin (2 min) and covered. Colon sections were screened (100x magnification) and the total number of tryptase positive cells in the mucosa was quantified using ImageJ 1.51 J8 (National Institutes of Health, USA) and expressed per mm^2.

Immunohistochemistry for PAR2, PAR4 and TRPA1

Distal colon samples were fixed in 4% paraformaldehyde and further processed for cryo-embedding. Cryo-sections (12µm) were thaw-mounted on poly-L-lysine-coated slides and dried at room temperature for 2 hours. Subsequently, these sections were permeabilized and blocked in 0,01M phosphate-buffered saline (PBS) containing 0,3% Triton X-100 and 10% normal horse serum for one hour at room temperature, after which they were incubated with primary antibodies for 16 hours at 4°C. All antibodies were diluted in 0,01M PBS containing 0,3% Triton X-100 and 1% bovine serum albumin. The following primary antibodies were used: rabbit anti-PAR4 (1/200, Alomone labs), rabbit

anti-PAR2 (1/100, Santa Cruz), rabbit anti-TRPA1 (1/1000, Abcam) and goat anti-CGRP (1/3000, Abcam). After several washing steps with 0,01M PBS, visualization was done using CY3-conjugated donkey anti-rabbit immunoglobulins (1/1000, Jackson ImmunoResearch) or CY5-conjugated donkey anti-goat immunoglobulins (1/1000, Jackson ImmunoResearch). Specificity was confirmed using negative controls and isotype controls. High-resolution images were obtained on a Leica TCS SP8 confocal laser scanning microscope and images were processed using the ImageJ software.

Proteolytic activities

To assess the activity of serine proteases in post-inflammatory visceral hypersensitivity, trypsin-like, chymotrypsin-like, neutrophil elastase, pancreas elastase and kallikrein activities were determined in colonic and fecal samples. Distal colon samples of approximately 50 mg were taken upon sacrifice, rinsed with Krebs solution, snap-frozen within 5 min and stored at -80 °C until further processing. The preparation started by crushing the colonic samples on dry ice using liquid nitrogen to avoid loss of activity due to temperature increase. Afterwards the samples were dissolved in lysis buffer (composition depending on assessed activity) for 15 minutes and centrifuged (4°C, 5 minutes, 12000 x g). The supernatant was collected and used immediately for the activity measurement. Lysis buffer for trypsin-like activity contained 1% octylglucoside (Roth, Germany) and 0,05% heparin (Sigma Life Science, Germany) in 50 mM Tris-HCl pH 7,4. The buffers for the other enzyme activities were all the same (1% octylglucoside in 50 mM Tris-HCl pH 7,4) except for the addition of 0.1% heparin in the buffer for chymotrypsin-like activity.

Boc-Gln-Ala-Arg-AMC (75 µM) and n-Tosyl-Gly-Pro-Arg-AMC (100 µM) (both Bachem) were used to explore the trypsin-like activity. Colonic supernatant was placed in a cold microtiterplate and preheated (37°C) substrate solutions in 50 mM Tris-HCl pH 8,0 were added to the samples to start the incubation. Fluorescence was measured kinetically for 20 minutes at 37°C on Tecan Infinite F200 Pro. The activities (U L^{-1}) were transformed to specific activities (U g^{-1}) using the protein concentration in the samples as determined by Bradford analysis. Chymotrypsin-like, neutrophil

elastase, pancreas elastase and kallikrein activities were measured using Suc-Ala-Ala-Pro-Phe-AMC (0,45 mM), Suc-Ala-Ala-Pro-Val-AMC (0,45 mM), Suc-Ala-Ala-Ala-AMC (0,45 mM) and H-Pro-Phe-Arg-AMC (0,45 mM) (all Bachem) in 50 mM Tris-HCl pH 8,0 respectively. The protocol for these activity experiments was identical to the one for trypsin-like activity except for a 5 minute pre-incubation of the samples.

Experiments to determine proteolytic activities in the fecal samples were performed in the same way except for the lysis buffer, which did not contain heparin or octylglucoside, and lysis time, which was 10 minutes instead of 15 minutes.

Statistical analysis

All data are presented as mean ± SEM. The statistical analysis was performed using SPSS Statistics (version 24.0, IBM). Results (VMR and compliance) were analyzed using a Generalized Estimating Equations (GEE) test with Least Significant Difference (LSD) post-hoc test. Inflammatory parameters and immunohistochemistry results were analyzed using a Two-way ANOVA with Student-Newman-Keuls (S-N-K) post-hoc test, as appropriate. Post-hoc tests were only carried out if F achieved $P<0.05$ and there was no significant variance in homogeneity. qPCR results were analyzed using unpaired Student's t-test or Two-way ANOVA with S-N-K post-hoc test. Proteolytic activities were analyzed using a Mann-Whitney U test. A p-value <0.05 was considered statistically significant. The graphs were made using GraphPad Prism 6.0. The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology(Curtis et al., 2015).

Materials

Active compounds

Nafamostat mesilate (Selleckchem®, also named FUT-175) is a broad-spectrum serine protease inhibitor, commercially available in Japan for the treatment of acute pancreatitis and disseminated intravascular coagulation(Isozaki et al., 2006). UAMC-00050 and UAMC-01162 are serine protease

inhibitors with a well-defined multi-target inhibition profile, developed by the Antwerp Drug Discovery Network (patent WO2007045496)(Joossens et al., 2007). These compounds slightly differ in both structural properties as well as in inhibitory potency; UAMC-00050 (*bis*(acetamidophenyl) guanidinophenylethylphosphonate) is a more potent inhibitor compared to UAMC-01162 with a highly similar structure, namely diphenyl guanidinophenylethylphosphonate. The inhibition profiles (displayed as IC_{50} values) of the three serine protease inhibitors used in the present study are provided in appendix ~~2~~1 and described in detail previously(van Soom et al., 2015).

Other chemicals and reagents

Heparin, hydrogen peroxide, 2,4,6-trinitrobenzenesulphonic acid (TNBS) and trypsin were purchased from Sigma-Aldrich (Steinheim, Germany). Ketamine (Ketalar®) was purchased from Pfizer (Pfizer, Puurs, Belgium). Xylazine (Rompun®) was purchased from Bayer (Bayer, Leverkusen, Germany). 0.9% sodium chloride was purchased from Braun (Diegem, Belgium). Pentobarbital 60 mg/ml (Nembutal®) was purchased from Ceva (Brussels, Belgium). The Krebs-Ringer solution has the following composition: 118.3 mM NaCl, 4.7 mM KCl, 1,2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 2 mM $NaCHO_3$, 0.026 mM CaEDTA and 11.1 mM glucose. Mouse anti-mast cell tryptase monoclonal antibody, rabbit anti-TRPA1 and goat anti-CGRP were ~~as~~ purchased from Abcam (Cambridge, UK). Goat anti-mouse IgG antibody, rat serum and Vectastain® avidin-biotin complex were obtained from Vector Laboratories (CA, USA). Rabbit anti-PAR4 was purchased from Alomone Labs (Jerusalem, Israel). Rabbit anti-PAR2 was obtained from Santa Cruz Biotechnology (Heidelberg, Germany). CY3-conjugated donkey anti-rabbit immunoglobulins and CY5-conjugated donkey anti-goat immunoglobulins were obtained from Jackson ImmunoResearch (Ely, UK). Octylglucoside was purchased from Roth (Wiesbaden, Germany). Boc-Gln-Ala-Arg-AMC, n-Tosyl-Gly-Pro-Arg-AMC, Suc-Ala-Ala-Pro-Phe-AMC, Suc-Ala-Ala-Pro-Val-AMC, Suc-Ala-Ala-Ala-AMC, H-Pro-Phe-Arg-AMC were obtained from Bachem (Bubendorf, Switzerland).

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., 2017).

RESULTS

Nafamostat mesilate decreases visceral hypersensitivity in a post-inflammatory setting

TNBS rats developed colitis on day 3, as demonstrated by the significantly increased colonoscopic inflammatory scores compared to controls (table 1A). At the day of the VMR, the post-inflammatory status of the animals was confirmed by colonoscopy, macroscopy, microscopy and colonic MPO activity.

Compared to control rats, vehicle-treated post-colitis rats showed significantly increased VMRs for ~~all~~ distension pressures (20-60mmHg~~)~~, indicating post-inflammatory visceral hypersensitivity (figure 2A). A single i.p. administration of nafamostat (0.01-10 mg/kg) 30 min before the VMR experiment, attenuated visceral sensitivity. In a dose of 0.01 mg/kg, VMRs were significantly decreased at 20-60 mmHg (figure 2A), whereas a dose of 0.1 mg/kg 1 mg/kg significantly lowered VMRs at 30-40-60 mmHg (figure 2B) and a dose of 1 mg/kg only affected VMRs at 20 and 30 mmHg (figure 2C). In the highest dose (10 mg/kg), no significant effect of nafamostat was observed in post-colitis rats (figure 2D). In control animals, the ~~most effective~~ dose of 0.1 mg/kg had no effect on visceral sensitivity (figure 2E). Nafamostat (0.1 mg/kg) had no effect on colonic compliance (figure 2F). Moreover, nafamostat treatment did not affect the inflammatory parameters compared to vehicle-treated controls (table 1A).

UAMC-00050 decreases visceral hypersensitivity in a post-inflammatory setting

On day 3, all TNBS rats displayed significantly higher colonoscopic inflammatory scores compared to controls, indicating the presence of a mild colitis (table 1B). At the day of the VMR, the post-inflammatory status of all animals was confirmed by colonoscopy and post-mortem inflammatory markers.

Rats in the post-colitis group displayed significantly higher VMRs compared to controls, confirming visceral hypersensitivity (figure 3A). A single i.p. administration with UAMC-00050 decreased VMRs in a dose-dependent manner in post-colitis animals: 0.01 mg/kg had no effect (figure 3A) whereas

0.1 mg/kg UAMC-00050 significantly decreased VMRs at 30-40-60 mmHg (figure 3B) and 1 mg/kg UAMC-00050 completely restored sensitivity to normal values (figure 3C). The most effective dose of 1 mg/kg UAMC-00050 had no effect on VMRs in control animals (figure 3D) and had no effect on colonic compliance (figure 3E). UAMC-00050 (0.01-1 mg/kg) had no effect on the inflammatory markers (table 1B~~2~~).

UAMC-01162 decreases visceral hypersensitivity in a post-inflammatory setting

A mild colitis was present in all TNBS animals, as confirmed by the significant higher colonoscopic inflammatory score at day 3 compared to controls (table 1C). At the end of the experiments, the post-inflammatory status of all animals was confirmed.

Vehicle-treated post-colitis rats displayed significantly higher VMRs compared to controls, demonstrating visceral hypersensitivity (figure 4A). A single i.p. injection of 1 mg/kg UAMC-01162 in post-colitis animals had no effect on VMR (figure 4A) whereas 2.5 mg/kg UAMC-01162 completely reversed visceral hypersensitivity (figure 4B). In control animals, no changes in visceral sensitivity were observed after a single i.p. injection with 2.5 mg/kg UAMC-01162 (figure 4C). UAMC-01162 did not alter colonic compliance (figure 4D) and had no effect on colonic inflammatory markers (table 1C).

mRNA quantification of serine proteases, protease-activated receptors and transient receptor potential ion channels

The selection of serine proteases for qPCR analysis was based on the inhibition profiles of the respective inhibitors. The relative mRNA expression of Tpsab1 (tryptase) was increased in the colon of post-colitis rats, while the expression of Plau (urokinase plasminogen activator), St14 (matriptase) and KLK8 (kallikrein 8) were comparable between control and post-colitis groups. KLK2 (kallikrein 2), KLK4 (kallikrein 4) and Ctsg (cathepsin G) could not be detected (table 2). Nafamostat, UAMC-00050 or UAMC-01162 did not affect serine protease expression at the mRNA level (table 2).

Recently, the implication of the serine protease trypsin has been shown in visceral hypersensitivity (Rolland-Fourcade et al., 2017). We therefore also investigated the colonic mRNA expression levels of the three trypsin isoforms: PRSS1 (trypsin-1 precursor), PRSS2 (trypsin-2 precursor), PRSS3 (trypsin-3 precursor). The mRNA expression level of PRSS1 was comparable in control and post-colitis rats (1.26 ± 0.28 vs 1.43 ± 0.40; n=12; ns), while PRSS3 was significantly upregulated in the post-colitis group (1.09 ± 0.31 vs 3.84 ± 1.20; n=12; p=0.03). PRSS2 was below the limit of detection (data not shown).

We also assessed colonic mRNA expression of receptors involved in the serine protease signaling cascade i.e. protease-activated receptors (PAR) and transient receptor potential ion channels (TRP)(Cenac, 2013). mRNA expression of PAR4 was significantly upregulated in colonic samples of post-colitis animals, while TRPV4 tended to be increased but this upregulation did not reach significance (p=0.074). No significant differences were detected for PAR2, TRPA1 and TRPV1 in the colon of control versus post-colitis animals (table 3).

qPCR experiments on DRGs were executed at the level of colonic splanchnic afferent nerves (T13-L2) and colonic pelvic afferent nerves (L6-S1). qPCR on DRG level T13-L2 revealed a clear trend towards upregulation of all PARs and TRPs in the post-colitis group, showing significant results for PAR4 and TRPA1. At DRG level L6-S1, a trend towards a lower mRNA expression of PARs and TRPs was seen in the post-colitis animals, showing significant results for PAR2 (table 3, *).

Immunohistochemistry for mast cell tryptase

The total number of mast cell tryptase positive cells per mm^2, quantified by immunohistochemistry in colonic mucosa, was significantly increased in post-colitis rats compared to control animals (figure 5). Similar to the mRNA results, treatment with serine protease inhibitors did not affect mast cell tryptase expression at the protein level (figure 5).

Immunohistochemistry for PAR2, PAR4, TRPA1

To investigate the potential involvement of the protease-PAR-TRP axis, as reviewed by Balemans *et al.* (Balemans et al., 2017), we determined the expression of PAR2, PAR4 and TRPA1 in nerve fibers in the lamina propria of the distal colon. CGRP-immunoreactive sensory nerve fibers showed a clear co-expression of PAR2 (figure 6A-B-C), whereas PAR4 appeared to be absent in these fibers (figure 6D-E-F). TRPA1, which is a downstream target of PAR2 signaling, was also co-expressed with CGRP in lamina propria nerve fibers (figure 6G-H-I).

Proteolytic activities

In order to assess the proteolytic activities in control vs post-colitis rats; trypsin-like, chymotrypsin-like, neutrophil elastase, pancreas elastase and kallikrein activities were determined in colon and fecal samples. Trypsin-like activity was found to be significantly upregulated in the colon of post-colitis rats compared to controls using both Boc-Gln-Ala-Arg-AMC (figure 7 A) and n-Tosyl-Gly-Pro-Arg-AMC (figure 7 B) as a substrate. Neutrophil elastase, pancreas elastase, kallikrein and chymotrypsin-like activities were not significantly different in colonic samples from post-colitis vs control animals (figure 7 C-F). In fecal samples, no significant differences could be detected between control and post-colitis rats (appendix 3 A-F).

DISCUSSION

The aim of this study was to elucidate the effects of serine protease inhibitors on post-inflammatory visceral hypersensitivity and their targets of action. Our results suggest that treatment with serine protease inhibitors reverses the visceral pain response to colonic distension in rats with post-inflammatory visceral hypersensitivity. Nafamostat significantly decreased visceral hypersensitivity, with a complete reversal in the lowest dose tested. Furthermore, both UAMC-00050 and UAMC-01162, two newly developed serine protease inhibitors [patent WO2007045496 (A1)](Joossens et al., 2007) significantly lowered visceral hypersensitivity in a dose-dependent way, completely restoring visceral sensitivity in the highest dose used. The serine protease inhibitors had no effect in healthy controls and did not affect colonic compliance, excluding an effect on viscoelastic properties of the colon.

Next, we tried to further unravel the specific serine proteases involved and their possible mode of action. Firstly, in order to define the serine proteases involved in a state of post-inflammatory visceral hypersensitivity, mRNA gene expression assays were performed. Colon tissue was tested for the expression of a panel of serine proteases, defined by those with an $IC_{50} < 10^{-6}M$ for the serine protease inhibitors tested in this study. Our data revealed a 4-fold increased mRNA expression of the serine protease tryptase as well as a significant upregulation of PRSS3 at the colonic level in post-colitis rats, while all other serine proteases tested in our panel were not significantly altered or below the detection limit in colon tissue. We confirmed this result at the protein level, by demonstrating a significantly increased number of mast cell tryptase positive cells in the colonic mucosa of post-colitis rats compared to controls. Our results thereby point towards an important role for the serine protease tryptase in post-inflammatory visceral hypersensitivity. Moreover, we could not show evidence for KLK2, KLK4, KLK8, cathepsin G and uPA at the colonic level.

These serine protease profile determinations could be of great importance towards the development of a new biomarker for IBS(Barbara, 2015). Previous studies showed an increased fecal

protease activity in IBS-D (serine proteases) and IBS-C (cysteine proteases) patients compared to healthy controls(Annahazi et al., 2013; Gecse et al., 2008; Roka et al., 2007), highlighting the potential of proteases as a diagnostic tool.

Interestingly, the three serine protease inhibitors did not affect mRNA/protein expression, suggesting an effect on the activity of the enzymes rather than on the expression. It is of great interest to determine the serine protease activity as well as the effect of the serine protease inhibitors hereon, but the currently available tools to study protease activity, such as the azocasein assay, are very unspecific(Edgington-Mitchell, 2015). The fluorogenic substrates used here allow to better characterize the proteolytic activities. Trypsin-like activity was found to be significantly increased in the colon of post-colitis rats compared to controls with both substrates, while no significant differences could be detected in fecal samples. However, more specific probes and/or inhibitors to directly assess the activity of individual proteases are essential for a more precise interpretation of the results. Edgington-Mitchell *et al.* recently demonstrated the efficacy of two fluorescent activity based probes detecting serine proteases *in vitro*, but their use was less valuable in two *in vivo* animal models for acute inflammation (pancreatitis and colitis)(Edgington-Mitchell et al., 2017). Optimization of these probes for *in vivo* use is of great importance when studying the role of serine proteases in several pathologies.

Remarkably, nafamostat showed its greatest potential at the lowest dose used pointing to a loss of specificity of the compound on visceral hypersensitivity: nafamostat 0.01 mg/kg was more effective than 0.1 mg/kg and 1 mg/kg and no effect was observed in a dose of 10 mg/kg. Amongst all of the examined proteases, the affinity of nafamostat is the highest for human tryptase ($9.53x10^{-11}$M). Thus, when used in a relatively low concentration, nafamostat is an extremely potent and selective inhibitor of human tryptase(Mori et al., 2003) suggesting that by using higher concentrations the selectivity for tryptase might ~~be lower~~ get lost, altering the protease profile ~~balance~~ in a more profound way.

Besides investigating the type of serine proteases involved in visceral hypersensitivity, we also studied the source of the proteases and site of action of the serine protease inhibitors. Sources of proteases in the gastrointestinal tract are diverse and include pancreas, microbiome, epithelial cells, neutrophils, macrophages and mast cells(Vergnolle, 2016). Our results provide evidence for the presence of tryptase in mast cells. Recently, the group of Vergnolle demonstrated the release of trypsin-3 by the intestinal epithelium and showed its involvement in visceral hypersensitivity(Rolland-Fourcade et al., 2017). This highlights that further study is needed towards the source of proteases implicated in visceral pain.

We hypothesize that the compounds used in our study directly inhibit serine proteases early in the signaling cascade, thereby preventing the proteases from activating PARs which on their turn are incapable of TRP sensitization thereby preventing visceral hypersensitivity. A recent review on TRP channels reports that pro-inflammatory mediators such as serotonin, histamine, bradykinins and proteases can activate various G-protein coupled receptors (GPCR) such as PAR receptors, thereby triggering TRP sensitization in visceral hypersensitivity (Balemans et al. 2017). To investigate this hypothesis a bit further in our model, we assessed the mRNA expression of PARs and TRPs which play a role in visceral hypersensitivity and serine protease downstream signaling pathways, at the colonic level and at DRGs. qPCR analysis revealed a significant upregulation of PAR4 in IBS rats at the colonic level and DRG T13-L2. A significant upregulation of TRPA1 was found in DRG T13-L2, while TRPV4 showed a tendency to increase in the colon of IBS rats. Interestingly no significant upregulations of PARs and/or TRPs were found in DRGs L6-S1, highlighting the importance of thoracolumbar spinal cord (T13-L2) in the processing of visceral pain signals(Christianson et al., 2006; Traub, 2000). These mRNA data support the involvement of both PARs and TRPs in the downstream pathways of serine proteases involved in post-inflammatory visceral hypersensitivity.

In order to reinforce our hypothesis on the involvement of PAR and TRP receptors in visceral hypersensitivity, immunohistochemical experiments were performed next to the qPCR experiments.

Increased expression of mast cell tryptase was observed both at the mRNA level as well as immunohistochemically in the colon of post-colitis rats compared to control animals. Tryptase is a PAR2 ligand and the involvement of both tryptase and the PAR2 receptor in visceral hypersensitivity has already been reported previously (Cenac et al., 2007; Vergnolle et al., 2001). We now demonstrate the presence of PAR2-immunopositive nerve fibers in the colon of post-colitis rats, for which double labeling with CGRP confirmed their sensory origin. We thus provide evidence at the protein level of the presence of both tryptase and PAR2 in the colon of post-colitis animals. Furthermore, we could also demonstrate the presence of TRPA1 in CGRP-positive nerve fibers in the lamina propria of the colon. Previously, Cattaruzza and colleagues already demonstrated that TRPA1 receptors mediate PAR2-induced visceral hypersensitivity (Cattaruzza et al., 2010). Thus, our results fit in the general hypothesis as mentioned earlier and described in the review by Balemans *et al*. (Balemans et al., 2017).

Interestingly, a downregulation of PAR4 was shown in colonic biopsies of IBS-D, IBS-C and post-infectious IBS patients(Han et al., 2012; Zhao et al., 2012). This discrepancy may be explained by a study of Annahazi *et al*(Annahazi et al., 2012). In a low-grade TNBS-colitis mouse model, PAR4 antagonism increases colorectal hyperalgesia. Their proposed mechanism is an endogenous activation of PAR4 (possibly by Cat-G), inducing a feedback antinociceptive effect. Moreover, PAR4 activation has been shown to result in antinociceptive effects as demonstrated by the inhibition of the excitability of mouse and rat colonic DRGs after the application of a PAR4-activating peptide (AP) (Asfaha et al., 2007; Karanjia et al., 2009), a decrease in carrageenan-induced inflammatory visceral hypersensitivity after an injection with a PAR4-AP (Asfaha et al., 2007) and a reduced visceral hypersensitivity in mice after an intracolonic administration with the PAR4-agonist AYPGKF-NH2 (Auge et al., 2009). We therefore hypothesize that the increased mRNA expression of PAR4 in the colon and DRG samples of post-colitis rats might be a consequence of post-inflammatory visceral hypersensitivity, rather than a cause. Besides, we were able to localize PAR-4 in epithelial cells as well as in myenteric and submucosal neurons.

The trend towards increased expression of TRPV4 in colon of post-inflammatory IBS rats is in line with the increased expression of TRPV4 in human colon biopsies in IBD patients(Fichna et al., 2012). However, to our knowledge, no such data are available for IBS patients. Also, the upregulation of TRPA1 in DRGs of rats with visceral hypersensitivity following TNBS-induced colitis is in line with the findings from Yang *et al.*(Yang et al., 2008). Also in a water-avoidance-stress-induced rat model for IBS, a significant upregulation of TRPA1 was seen in DRGs(Yu et al., 2010).

In summary, we investigated the effect of three different serine protease inhibitors in a rat model for post-inflammatory visceral hypersensitivity. After a single administration of either of these compounds, a decrease in visceral pain was proven in post-colitis rats but no effect was observed in healthy controls. Our work differs from previous studies in three key aspects. Firstly, we opted for a direct *in vivo* treatment strategy, with a single intraperitoneal injection of the animals, while preceding studies employed an *in vitro* treatment strategy(Cenac et al., 2007; Wang et al., 2015). Secondly, we are the first to demonstrate a positive outcome after a symptomatic treatment instead of a preventive treatment in earlier studies(Cenac et al., 2007; Wang et al., 2015; Zhao et al., 2011). Thirdly, the animal models used are different. Previous studies employed intracolonic administration in mice of supernatants from IBS patient(Cenac et al., 2007; Wang et al., 2015) or acute stress in rats(Zhao et al., 2011) to induce visceral hypersensitivity whereas we used an inflammation-triggered post-colitis model based on chemically-induced colitis in rats. We provide further evidence for the role for the serine proteases tryptase and trypsin-3 in this post-inflammatory rat model for IBS, evidenced by elevated mRNA expression and an increased number of mast cell tryptase positive cells. Moreover, the increased tryptase expression at the colonic level of post-colitis rats, increased trypsin-like serine protease activity in the colon of post-colitis rats, the localization of PAR2 and TRPA1 in the sensory nerve fibers in the colon together with the increased TRPA1 mRNA expression at the DRG level in post-colitis rats, suggest their possible involvement in post-inflammatory visceral hypersensitivity.

Our results indicate that serine protease inhibition represents an interesting new treatment strategy for abdominal pain in IBS patients. Regarding the search for new biomarkers in IBS patients, our study points towards serine proteases and more specifically tryptase as a possible candidate.

proteases - http://www.guidetopharmacology.org/GRAC/FamilyDisplayForward?familyId=759

protease-activated receptors - http://www.guidetopharmacology.org/GRAC/FamilyDisplayForward?familyId=59

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TABLES

Table 1. Inflammatory parameters of nafamostat mesilate, UAMC-00050 and UAMC-01162 in control and post-colitis rats

A. Nafamostat mesilate

Group	Drug	N	Colonoscopy (0-19)		Macroscopy	Microscopy	MPO activity
			Day 3	Day VMR			
Control	Vehicle	8	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.4 ± 0.2	0.9 ± 0.3
	0.1 mg/kg	8	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.6 ± 0.4	0.8 ± 0.3
Post-colitis	Vehicle	8	5.8 ± 0.8*	0.0 ± 0.0	0.0 ± 0.0	0.8 ± 0.3	1.1 ± 0.3
	0.01 mg/kg	8	6.6 ± 0.7*	0.0 ± 0.0	0.0 ± 0.0	1.0 ± 0.5	1.5 ± 0.5
	0.1 mg/kg	8	7.0 ± 0.5*	0.0 ± 0.0	0.0 ± 0.0	0.4 ± 0.2	1.7 ± 0.4
	1 mg/kg	8	5.0 ± 0.4*	0.0 ± 0.0	0.0 ± 0.0	0.6 ± 0.4	1.4 ± 0.5
	10 mg/kg	7	6.0 ± 0.7*	0.0 ± 0.0	0.1 ± 0.1	0.7 ± 0.7	2.1 ± 0.5

B. UAMC-00050

Group	Drug	N	Colonoscopy (0-19)		Macroscopy	Microscopy	MPO activity
			Day 3	Day VMR			
Control	Vehicle	7	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.2	0.9 ± 0.2
	1 mg/kg	6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.5 ± 0.1
Post-colitis	Vehicle	8	5.4 ± 0.7*	0.0 ± 0.0	0.0 ± 0.0	0.5 ± 0.2	1.6 ± 0.5
	0.01 mg/kg	7	7.0 ± 0.9*	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.2	1.2 ± 0.5
	0.1 mg/kg	6	5.3 ± 0.9*	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.2	1.8 ± 0.9
	1 mg/kg	9	4.3 ± 0.6*	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1	1.2 ± 0.6

C. UAMC-01162

Group	Drug	N	Colonoscopy (0-19)		Macroscopy	Microscopy	MPO activity
			Day 3	Day VMR			
Control	Vehicle	8	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.1	0.9 ± 0.3
	2.5 mg/kg	8	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.1	0.6 ± 0.2
Post-colitis	Vehicle	8	6.5 ± 0.9*	0.0 ± 0.0	0.0 ± 0.0	0.6 ± 0.2	0.9 ± 0.4
	1 mg/kg	8	6.4 ± 0.8*	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.2	1.3 ± 0.2
	2.5 mg/kg	8	7.9 ± 0.9*	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.1	1.0 ± 0.2

Data are presented as mean ± SEM. Two-way ANOVA followed by S-N-K post-hoc test. No significant interaction, significant effect of the factor 'group' for colonoscopy at day 3; *$p<0.05$; no significant effect of the factor 'drug'. MPO; myeloperoxidase; N; number; VMR; visceromotor response.

Table 2. Relative mRNA expression of serine proteases in colon samples –nafamostat

A. Nafamostat mesilate

Gene	Control		Post-colitis	
	Vehicle	NFM 0.1 mg/kg	Vehicle	NFM 0.1 mg/kg
Tryptase αβ1	1.30 ± 0.37	1.32 ± 0.45	4.65 ± 1.07*	4.26 ± 1.41*
Matriptase	1.07 ± 0.15	1.25 ± 0.17	1.00 ± 0.16	1.05 ± 0.15
Cathepsin G	<LOD	<LOD	<LOD	<LOD
uPA	1.12 ± 0.21	1.77 ± 0.33	1.43 ± 0.31	1.07 ± 0.23
Kallikrein 2	<LOD	<LOD	<LOD	<LOD
Kallikrein 4	<LOD	<LOD	<LOD	<LOD
Kallikrein 8	1.21 ± 0.24	2.02 ± 0.57	1.18 ± 0.18	1.67 ± 0.63
	n=8	n=8	n=8	n=8

B. UAMC-00050

Gene	Control		Post-colitis	
	Vehicle	UAMC-00050	Vehicle	UAMC -00050
Tryptase αβ1	1.30 ± 0.37	3.14 ± 1.19	4.65 ± 1.07*	2.63 ± 0.44
Matriptase	1.07 ± 0.15	0.88 ± 0.09	1.00 ± 0.16	1.00 ± 0.13
Cathepsin G	<LOD	<LOD	<LOD	<LOD
uPA	1.12 ± 0.21	1.43 ± 0.31	1.43 ± 0.31	1.23 ± 0.19
Kallikrein 2	<LOD	<LOD	<LOD	<LOD
Kallikrein 4	<LOD	<LOD	<LOD	<LOD
Kallikrein 8	1.21 ± 0.24	1.50 ± 0.37	1.18 ± 0.18	1.28 ± 0.21
	n=8	n=6	n=8	n=7

C. UAMC-01162

Gene	Control		Post-colitis	
	Vehicle	UAMC-01162	Vehicle	UAMC-01162
Tryptase αβ1	1.29 ± 0.39	1.11 ± 0.37	2.27 ± 1.00	0.75 ± 0.22
Matriptase	1.05 ± 0.10	0.65 ± 0.11*	0.71 ± 0.11*	0.64 ± 0.07*
Cathepsin G	<LOD	<LOD	<LOD	<LOD
uPA	1.09 ± 0.16	1.09 ± 0.20	1.08 ± 0.16	1.07 ± 0.12
Kallikrein 2	<LOD	<LOD	<LOD	<LOD
Kallikrein 4	<LOD	<LOD	<LOD	<LOD
Kallikrein 8	1.19 ± 0.21	1.09 ± 0.12	0.91 ± 0.20	0.88 ± 0.16
	n=10	n=10	n=10	n=10

Data are expressed as relative mRNA expression and presented as mean ± sem for n=8 per group (NFM), n=8 per group (control/post-colitis + vehicle), n=6 per group (control + UAMC-00050), n=7/group (post-colitis + UAMC-00050), n=10 per group (UAMC-01162) . Two-way ANOVA followed by One-way ANOVA + LSD post-hoc test if applicable. A: Significant effect of the factor 'group' *p<0.05 for tryptase αβ1. No significant effect of the factor 'drug'. No significant interaction. B: No significant effect of the factor 'group'. No significant effect of the factor 'drug'. Significant interaction for tryptase αβ1. *p<0.05 significantly different from "control + vehicle". C: No significant effect of the factor 'group'. No significant effect of the factor 'drug'. Significant interaction for matriptase *p<0.05 significantly different from "control + vehicle". NFM: nafamostat mesylate; LOD; limit of detection.

Table 3. Relative mRNA expression of PARs and TRPs in colonic, DRG T13-L2 and DRG L6-S1 samples.

Gene	Colon		DRG T13-L2		DRG L6-S1	
	Control	Post-colitis	Control	Post-colitis	Control	Post-colitis
PAR2	1.05 ± 0.11	0.90 ± 0.14	1.07 ± 0.11	1.31 ± 0.08	1.07 ± 0.13	0.75 ± 0.04*
PAR4	0.94 ± 0.15	1.87 ± 0.30*	1.03 ± 0.08	1.60 ± 0.17*	1.05 ± 0.12	1.21 ± 0.25
TRPA1	1.09 ± 0.19	1.27 ± 0.28	1.04 ± 0.08	1.40 ± 0.12*	1.10 ± 0.15	0.81 ± 0.09
TRPV1	1.03 ± 0.10	0.81 ± 0.15	1.04 ± 0.08	1.19 ± 0.07	1.07 ± 0.12	1.49 ± 0.20
TRPV4	1.04 ± 0.11	1.73 ± 0.36[a]	1.10 ± 0.18	1.48 ± 0.17	1.05 ± 0.11	0.88 ± 0.10
	n=8	n=8	n=12	n=12	n=12	n=12

Data are expressed as relative mRNA expression and presented as mean ± sem for n=8 per group (colon) and n=12 per group (DRG). Independent samples T-test. *p<0.05 significant effect of the factor "group"; [a]p=0.074. DRG; dorsal root ganglia; PAR; protease-activated receptor; TRPA;

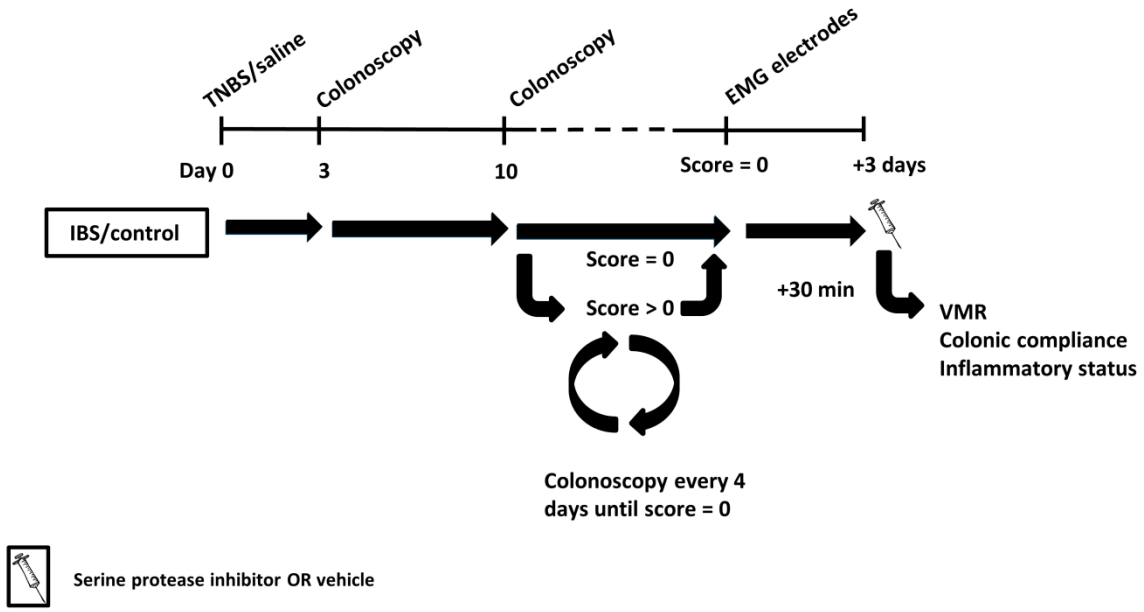


Figure 1. Overview of the experimental design. On day 0, rats received an intrarectal administration with TNBS (colitis) or saline (control). The severity of colitis and the mucosal healing were monitored *in vivo* using colonoscopy: on day 3 to confirm the presence of colitis and from day 10 onwards every 4 days until complete mucosal healing. Hereafter, EMG electrodes were implanted and 3 days later all experiments were conducted after a single i.p. injection with vehicle/serine protease inhibitor. EMG; electromyographic; IBS; irritable bowel syndrome; TNBS; 2,4,6-trinitrobenzenesulfonic acid; VMR; visceromotor response.

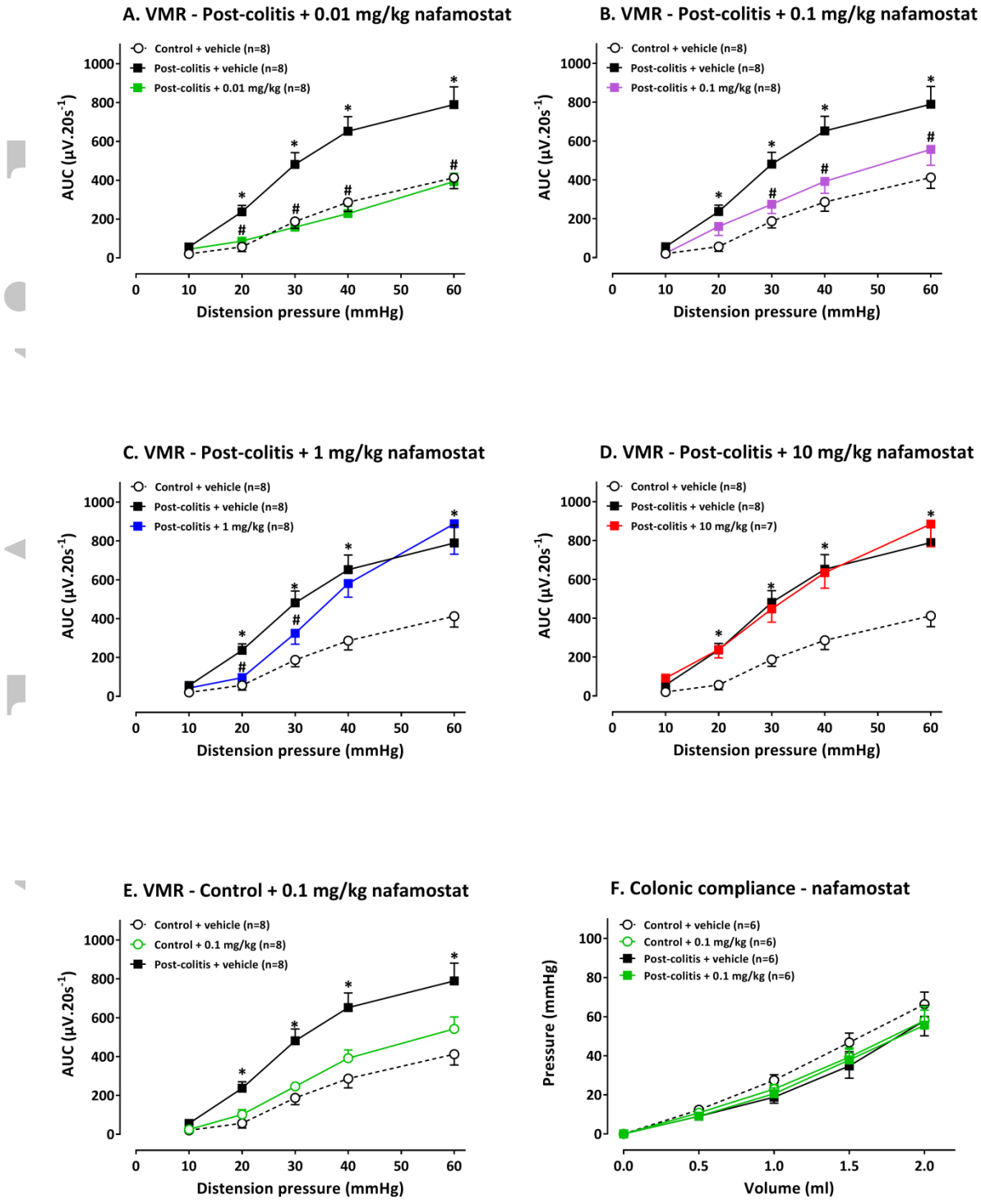


Figure 2. The effect of nafamostat mesilate (0.01-10 mg/kg) and its vehicle (water for injection) on VMRs (n=8 per group, n=7 per group for 10 mg/kg) and colonic compliance (n=6 per group) in post-colitis and control rats. The statistical analysis was performed on the complete dataset, but separate graphs were made for each dose for the purpose of clarification. Data are presented as mean ± SEM. Generalized Estimating Equations (GEE) + LSD post-hoc test; *p<0.05; significantly different from control + vehicle. #p<0.05; significantly different from post-colitis + vehicle.

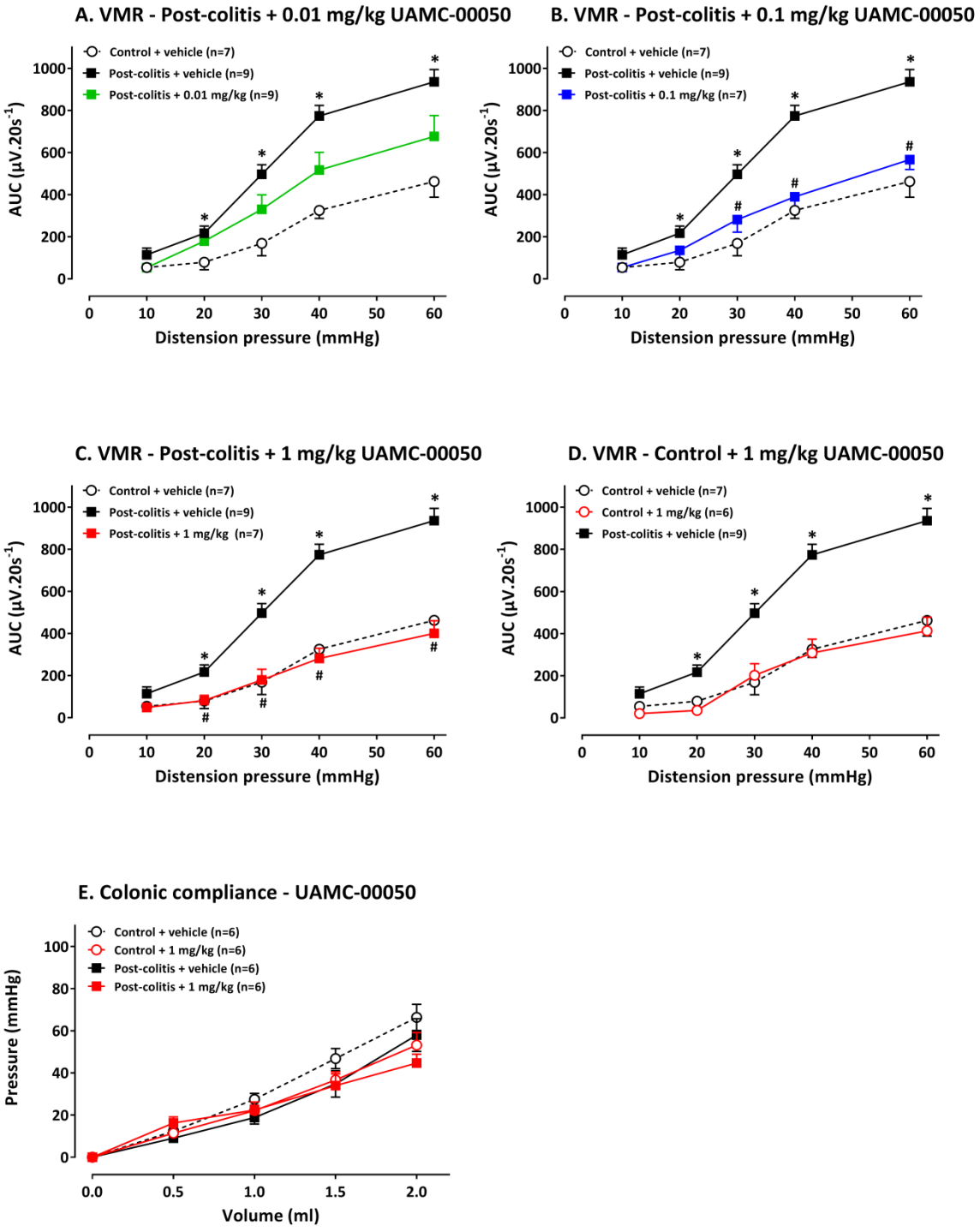


Figure 3. The effect of UAMC-00050 (0.01-1 mg/kg) and its vehicle (5% DMSO) on VMRs (control + 1 mg/kg; n=6 per group, control + vehicle, post-colitis + 0.1 mg/kg, post-colitis + 1 mg/kg; n=7 per group, post-colitis + vehicle, post-colitis + 0.01 mg/kg; n=9 per group) and colonic compliance (n=6 per group) in post-colitis and control rats. The statistical analysis was performed on the complete dataset, but separate graphs were made for each dose for the purpose of clarification. Data are presented as mean ± SEM. Generalized Estimating Equations (GEE) + LSD post-hoc test; *p<0.05; significantly different from control + vehicle. #p<0.05; significantly different from post-colitis + vehicle.

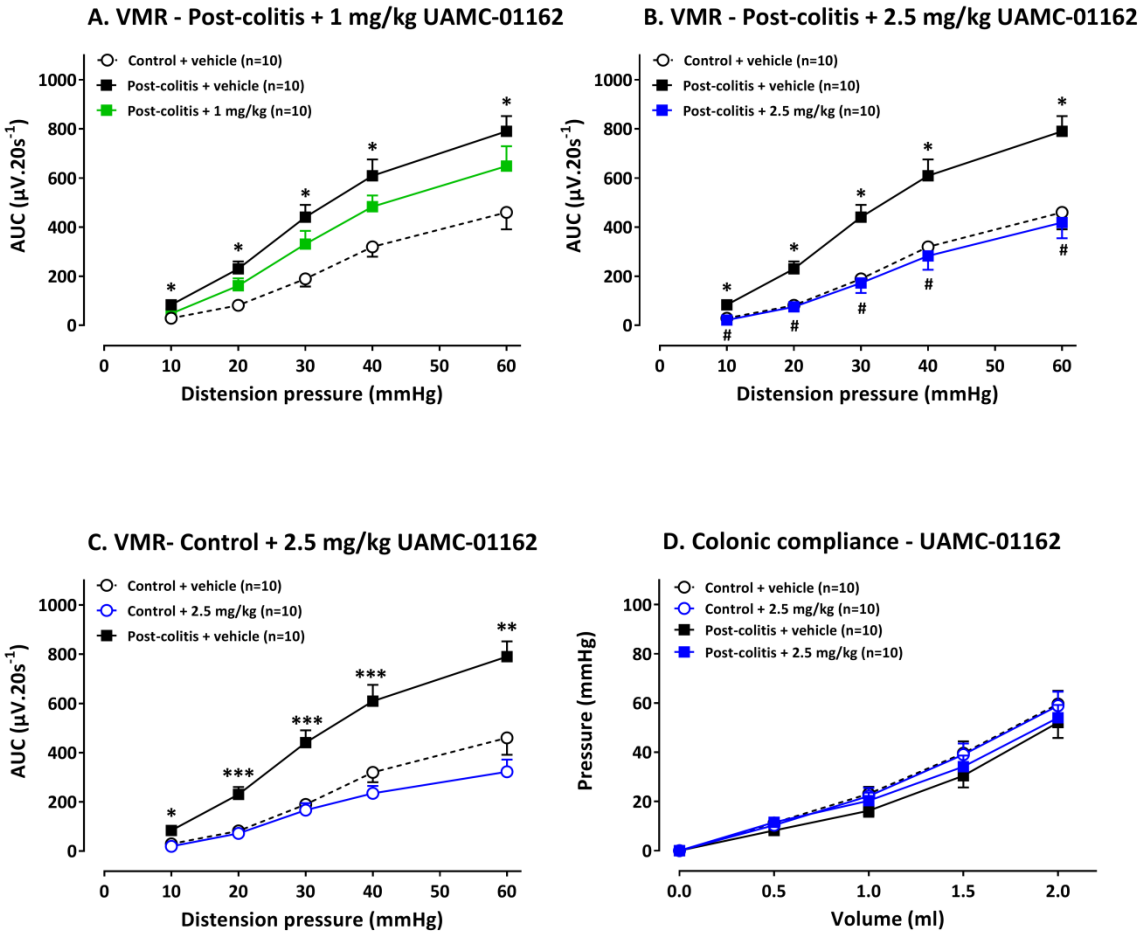


Figure 4. The effect of UAMC-01162 (1-2.5 mg/kg) and its vehicle (5% DMSO) on VMRs and colonic compliance in post-colitis and control rats (n=10 per group). The statistical analysis was performed on the complete dataset, but separate graphs were made for each dose for the purpose of clarification. Data are presented as mean ± SEM. Generalized Estimating Equations (GEE) + LSD post-hoc test; *p<0.05; significantly different from control + vehicle. #p<0.05; significantly different from post-colitis + vehicle.

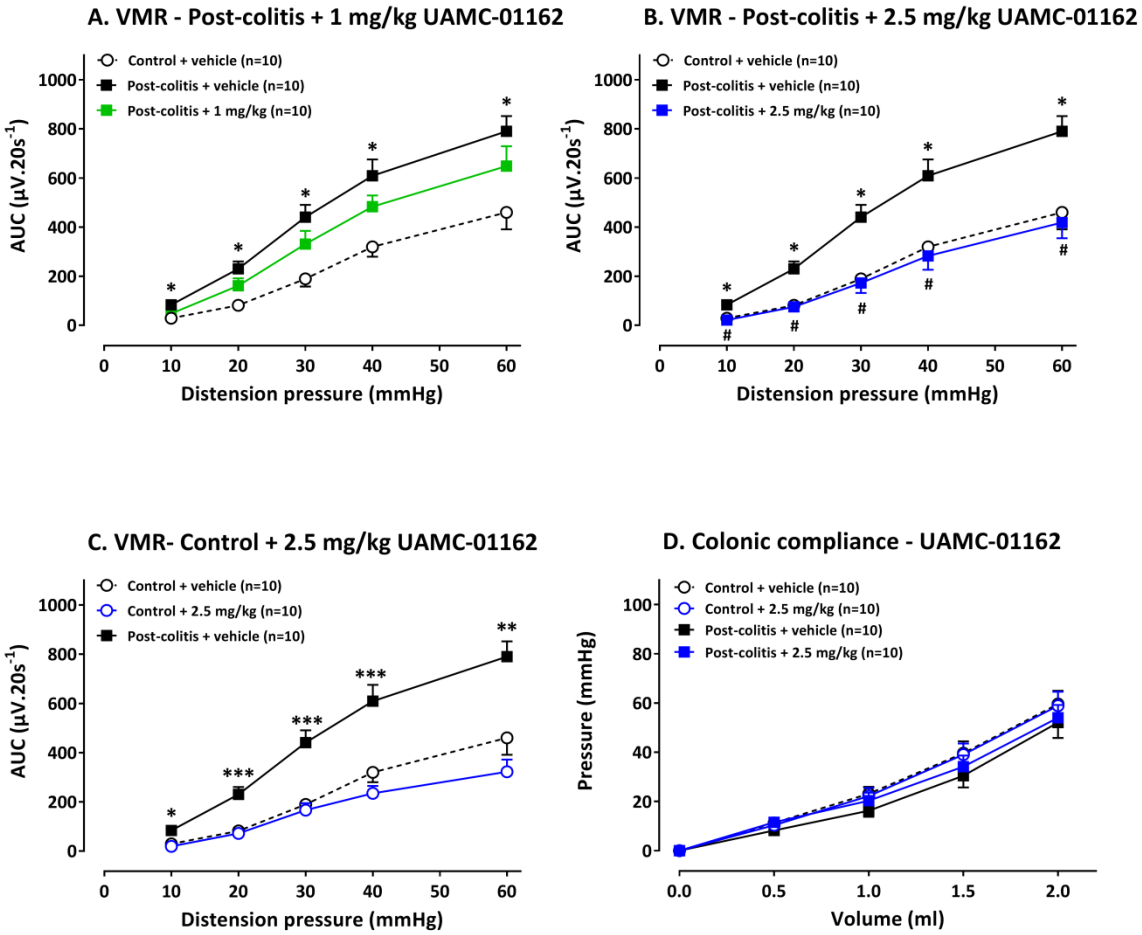


Figure 5. Immunohistochemistry with mast cell tryptase antibody in rat colon. A. Representative images of the colonic mucosa of a control and a post-colitis animal with mast cell tryptase (arrow). B, C, D. The number of tryptase-positive mast cells per mm² in the colonic mucosa of control and post-colitis rats with or without treatment with a serine protease inhibitor. Two-way ANOVA; n=8 per group; *p<0.05; significant effect of the factor 'group'; no significant effect of the factor 'drug'; no significant interaction between the factors 'group' and 'drug'.

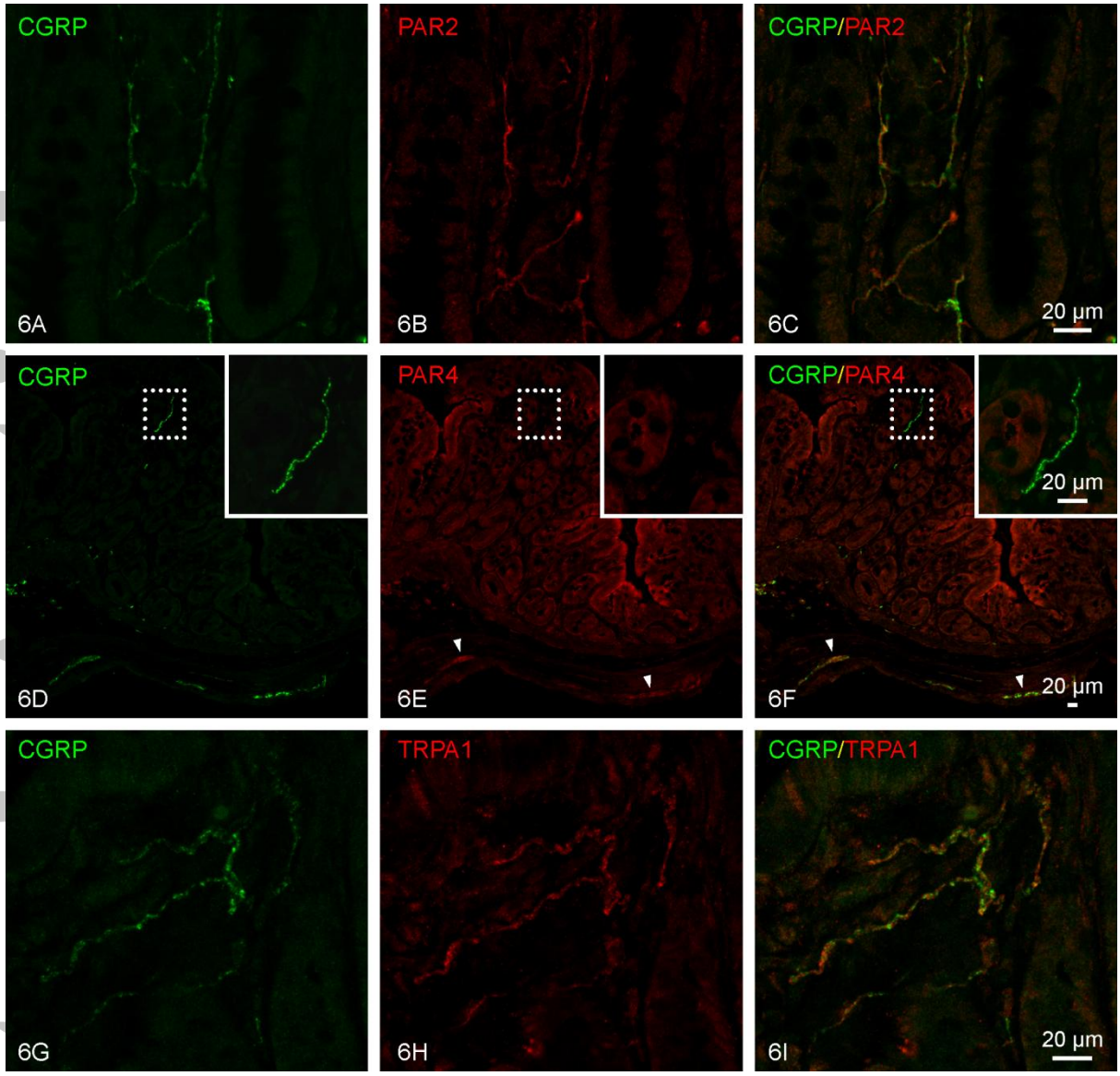


Figure 6. Immunohistochemical localization of PAR2, PAR4, TRPA1 in sensory nerve fibers of the distal colon. A-C. Representative image showing co-localization of PAR2 (red) in CGRP-immunopositive nerve fibers (green). D-F. Representative image showing the presence of PAR4 immunoreactivity (red) in the colonic epithelium and in enteric nerve plexuses (arrowheads), but not in the CGRP-immunoreactive nerve fiber population (inset). G-I. Representative image showing co-localization of TRPA1 (red) in CGRP-immunopositive nerve fibers (green).

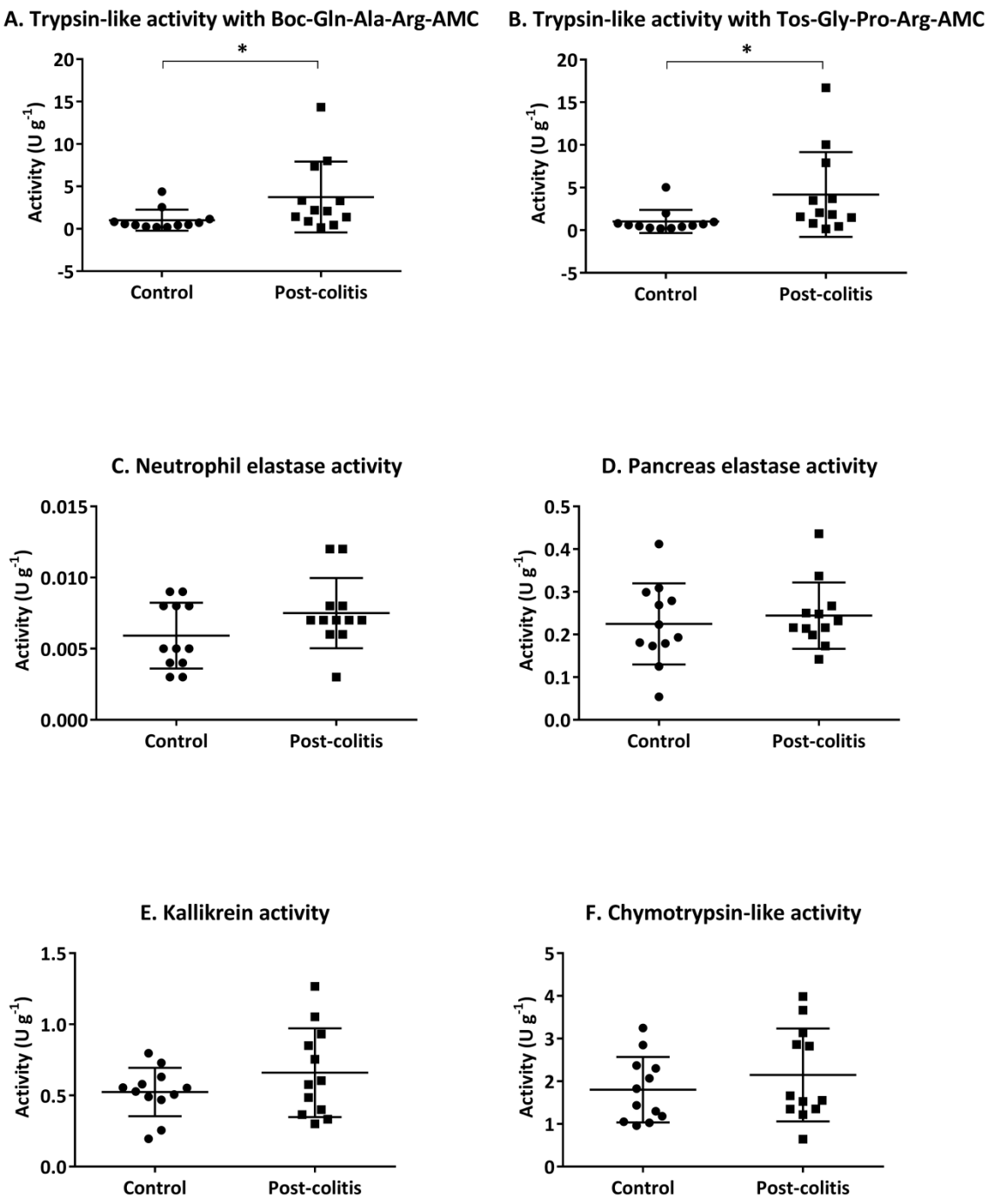


Figure 7. Proteolytic activities determined in colon samples. Trypsin-like activity was measured using Boc-Gln-Ala-arg-AMC and n-Tosyl-Gly-Pro-Arg-AMC. To determine the neutrophil elastase, pancreas elastase, kallikrein and chymotrypsin-like activity, Suc-Ala-Ala-Pro-Val-AMC, Suc-Ala-Ala-Ala-AMC, H-Pro-Phe-Arg-AMC and Suc-Ala-Ala-Pro-Phe-AMC were used respectively. Data are presented as mean ± SEM. Mann Whitney U-test; * $p<0.05$; n=12 per group.

APPENDIX 1

Taqman primers used for qPCR analysis of colonic and DRG samples.

Protein	Gene ID
Tryptase αβ1	Rn00570928_m1
Matriptase	Rn00586242_m1
Cathepsin G	Rn01489144_g1
Urokinase plasminogen activator	Rn00565261_m1
Kallikrein 2	Rn00820615_m1
Kallikrein 4	Rn01498534_g1
Kallikrein 8	Rn01476995_m1
PAR2	Rn00588089_m1
PAR4	Rn00587480_m1
TRPA1	Rn01473803_m1
TRPV1	Rn00583117_m1
TRPV4	Rn00576745_m1
GAPDH	Rn01775763_g1
β-actin	Rn00667869_m1

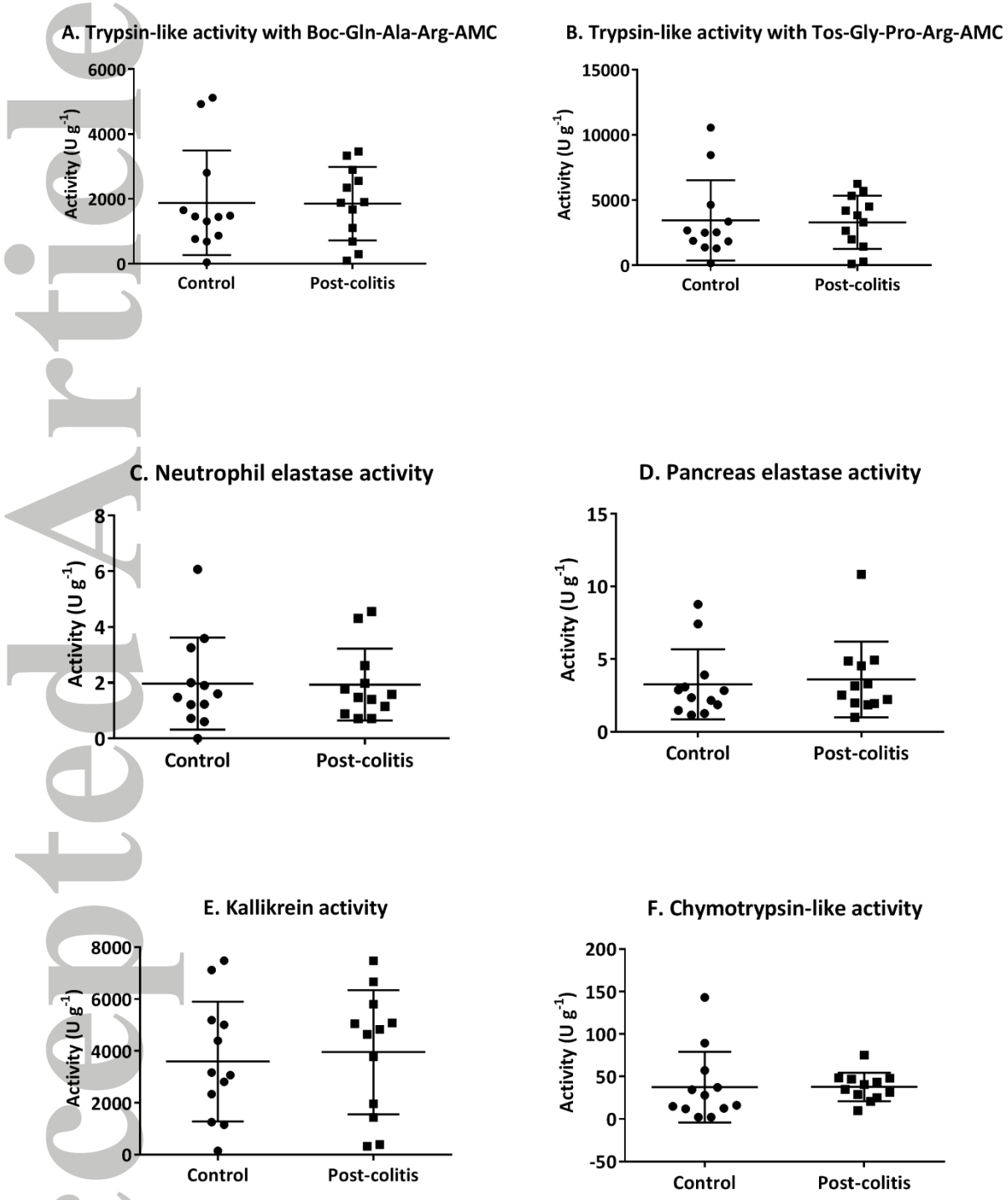
APPENDIX 2

Inhibitory profiles for the serine protease inhibitors UAMC-00050, UAMC-01162 and nafamostat mesilate.

Proteases	UAMC-00050 IC_{50} (μM)	UAMC-01162 IC_{50} (μM)	Nafamostat mesilate Est. IC_{50} (μM)
uPA	0.0042	0.0031	< 0.001
tPA	7	23	> 2.5
plasmin	0.9	13	± 0.05
thrombin	0.39	17	± 0.5
FXa	100	± 250	± 2
FXIIa	1.61	> 2.5	± 0.1
matriptase	0.0025	0.083	< 0.001
tryptase	0.028	0.093	< 0.001
cathepsin G	0.12	0.33	± 0.25
HNE	> 2.5	> 2.5	> 2.5
plasma kallikrein	> 2.5	> 2.5	± 0.005
KLK1	> 2.5	> 2.5	± 1
KLK2	0.11	3	± 0.1
KLK4	0.0017	0.009	± 0.005
KLK8	0.0016	0.028	± 0.015
AChE	> 20	> 10	> 2.5

Data are presented as mean ± SEF. Est.; estimated, FXa; factor Xa, FXIIa; factor XIIa, HNE; human neutrophil elastase, IC_{50}; half maximal inhibitory concentration, KLK; kallikrein, SEF; standard error on the fit, tPA; tissue plasminogen activator, uPA; urokinase plasminogen activator.

APPENDIX 3



Proteolytic activities determined in fecal samples. Trypsin-like activity was measured using Boc-Gln-Ala-arg-AMC and n-Tosyl-Gly-Pro-Arg-AMC. To determine the neutrophil elastase, pancreas elastase, kallikrein and chymotrypsin-like activity, Suc-Ala-Ala-Pro-Val-AMC, Suc-Ala-Ala-Ala-AMC, H-Pro-Phe-Arg-AMC and Suc-Ala-Ala-Pro-Phe-AMC were used respectively. Data are presented as mean ± SEM. Mann Whitney U-test; n=12.