THE SYNTHETIC HYDROXYPROLINE-CONTAINING COLLAGEN ANALOGUE (GLY-PRO-HYP)₁₀ AMELIORATES ACUTE DSS COLITIS

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Received: June 11, 2012; Accepted: June 17, 2012

In experimental models of and humans with intestinal inflammation, increased levels of the matrix-degrading gelatinases MMP-2 and -9 in inflamed tissues can be detected. The synthetic collagen analogue (Gly-Pro-Hyp)₁₀, (GPO)₁₀, has been identified as a relevant binding structure for proMMP-2/-9 and promotes enzymatic activity of proMMP-2. Since targeted MMP strategies might offer promising anti-inflammatory treatment options, we for the first time studied *in vivo* actions exerted by (GPO)₁₀ applying an acute dextrane sulfate sodium (DSS) induced colitis model. Seven-day intraperitoneal (GPO)₁₀ treatment ameliorated clinical symptoms and histopathological colonic changes as compared to placebo controls with severe colitis. (GPO)₁₀-treated mice displayed a diminished influx of neutrophils, and T- and B-lymphocytes into their colonic mucosa whereas numbers of regulatory T-cells and regenerative cells were higher as compared to placebo controls. Furthermore, IL-6 secretion was down-regulated in *ex vivo* colonic biopsies derived from (GPO)₁₀-treated mice whereas higher concentrations of the anti-inflammatory cytokine IL-10 in extra-intestinal compartments such as MLN and spleen could be detected. Strikingly, influx of inflammatory cells into lungs was abolished following (GPO)₁₀ application. We therefore propose (GPO)₁₀ as a promising effective and safe treatment option of intestinal and extra-intestinal inflammatory conditions in humans.

Keywords: (GPO)₁₀, matrix metalloproteinases, gelatinase, proMMP-2, extracellular matrix, acute DSS colitis, intestinal inflammation, pro-inflammatory cytokines, IL-10, proliferation, *in vivo*, extra-intestinal immune responses, lung, human IBD, crinopathy

Abbreviations: (GPO)₁₀, (Gly-Pro-Hyp)₁₀; PBS, phosphate buffered saline; DSS, dextrane sulfate sodium; p.i., post induction; MMP, matrix metalloproteinase; IBD, inflammatory bowel disease; i.p., intraperitoneal; p.o., peroral; MT-MMP, membrane-type matrix metalloproteinase; TIMP, tissue inhibitor of matrix metalloproteinases; TNF, tumor necrosis factor; IFN, interferon, Treg, regulatory T cell; Th, T helper; IL, interleukin; SPF, specific pathogen-free; HE, hematoxylin eosin; MLN, mesenteric lymphnodes

Introduction

Matrix metalloproteinases (MMP) comprise a heterogeneous family of 25 zinc- and calcium dependent endopeptidases which, with respect to their substrate specificity, can be categorized into collagenases (MMP-1, -8, -13, -18), gelatinases (MMP-2, -9), stromelysins (MMP-3, -7, -10, -11), elastase (MMP-12), and membrane-type matrix metalloproteinases (MT-MMP-1 through -5) [1–4]. MMPs are tightly controlled by a balance of activators (e.g. pro-inflammatory molecules such as IL-1, IL-6, TNF- α) and inhibitors (tissue inhibitors of matrix metalloproteinases (TIMPs), anti-inflammatory cytokines such as IL-4, IL-10, TGF- β) and contribute to synthesis and degradation of extracellular matrix, cytokine activation, and ligand

shedding [5, 6]. Under physiological conditions, MMPs are involved in embryonic development and differentiation, and proliferation and regeneration of tissues such as wound healing [1–4]. A disbalanced MMP expression, however, can run into pathological processes such as inflammation and tissue destruction. In experimental models of Th1-type inflammation (e.g. acute ileitis and colitis), [7–10] as well as in humans with inflammatory bowel diseases (IBD) such as Crohn's disease and ulcerative colitis [11–16], increased levels of the gelatinases MMP-2 and MMP-9 in inflamed tissue sites associated with increased mRNA levels of the pro-inflammatory cytokines IL-1, IL-6, and TNF- α could be detected. Interestingly, MMPs are known to shed biologically active IL-1, IL-6, and TNF- α molecules from the surfaces of effector cells such

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as macrophages, which, in turn, induce MMP expression from immune, epithelial, and parenchymal cells [17, 18].

Extracellular deposition of growth factors, cytokines, and enzymatically inactive proforms of MMPs (proMMP) that makes them rapidly and locally available is a phenomenon called crinopexy [19]. We identified the rigid collagen triple helix, a secondary structure element of collagens, exemplified by the collagen analog (Gly–Pro–Hyp)₁₀ – (GPO)₁₀ –, as a relevant binding structure for proMMP-2/-9 in the extracellular matrix [20]. The tissue distribution and substrate specificity of the gelatinases MMP-2 and MMP-9 are defined by their collagen-binding domains which enhance the interaction with substrate and non-substrate molecules. We could demonstrate that collagenous peptides like the alpha 2 chain of collagen VI or the synthetic peptide (GPO)₁₀ act on MMP-2/-9 collagen-binding domain-exosites thus releasing and activating the collagen-sequestered proMMPs from their extracellular depot [20, 21]. In very recent work, we demonstrated that this (GPO)₁₀–proMMP-2 interaction also impacts cellular responses in cell culture experiments (Freise et al., this issue of EUJMI). In IBD, MMP-2 is stored within the extracellular matrix of the gut submucosa, and MMP-9 is localized pericellularly in the lamina propria [22, 23]. Thus, targeted therapies against specific MMPs should be highly promising in treating intestinal inflammatory disorders with respect to clinical efficacy and rather little adverse side effects which might be due to unselective blockage of MMPs. We were therefore interested in potential beneficial effects of (GPO)₁₀ in acute DSS colitis. In the study presented here, we demonstrate that in mice treated with (GPO)₁₀ intraperitoneally i) clinical colitis pathology was ameliorated, ii) histopathological changes in the colon were less severe which was parallel by iii) less secretion of the pro-inflammatory cytokine IL-6 in their colon whereas iv) concentrations of the anti-inflammatory cytokine IL-10 in extraintestinal compartment such as MLN and spleen were increased. Furthermore, v) influx of pro-inflammatory immune cell influx into mucosa and submucosa was reduced whereas vi) numbers of Tregs and regenerative cells in the colon were increased. Finally, vii) influx of inflammatory cells into extra-intestinal tissue sites such as lungs was abolished following (GPO)₁₀ i.p.. We therefore propose (GPO)₁₀ as a promising future option for anti-inflammatory/immunomodulatory treatment options of intestinal and extra-intestinal inflammatory conditions in humans.

Materials and methods

Ethics statement

All animal experiments were conducted according to the European Guidelines for animal welfare (2010/63/EU) with approval of the commission for animal experiments headed by the “Landesamt für Gesundheit und Soziales” (LaGeSo, Berlin, Germany). Animal welfare was monitored twice daily by assessment of clinical conditions.

Mice and colitis induction

Female C57BL/6j wildtype mice were bred and maintained under specific pathogen-free (SPF) conditions in the Forschungsinstitut für Experimentelle Medizin (FEM, Charité, Berlin, Germany). For colitis induction, mice 3 months of age were treated with 3.5% (wt/vol) DSS (40 kDa, MP Biomedicals, Illkirch, France) in drinking water *ad libitum* for 6 days. Prior necropsy, mice received water without DSS for 24 h. The intake of the DSS solution was controlled, and mice were weighed daily. Mice without colitis induction served as negative controls.

Determination of clinical scores of large intestinal shortening

Total clinical scores with a maximum of 12 were generated daily by combined data of weightloss, occurrence of blood in stool (as determined by the Guajak method using Haemocult™, Beckman Coulter/PCD, Krefeld, Germany), and stool consistency, as described [24, 25]. The relative shortening of the colon was calculated by dividing the difference of the mean lengths of the large intestines from age- and sex-matched control mice minus the length of mice subjected to DSS at day 7 p.i. (post induction) and then multiplied by 100 over the mean colon length of naïve control mice (relative shortening in length = (mean d0–d7 p.i.) × 100/mean d0). Results were expressed as % shortage.

Treatment with (GPO)₁₀

The triple helical collagen analogue (GPO)₁₀ – H–Gly–Cys–Hyp–(Gly–Pro–Hyp)₁₀–Gly–Cys–Hyp–Gly–NH₂ – was synthesized and characterized as described previously [20, 26]. Mice (with and without DSS induction) were treated perorally (p.o.) by gavage or intraperitoneally (i.p.) twice daily with (GPO)₁₀ (2 mg/kg body weight/day) dissolved in 0.3 ml PBS starting at day 0 for 7 days until necropsy. PBS treated animals (0.3 ml perorally twice daily for the respective period of time) served as placebo controls.

Sampling procedures and histologic scoring

Mice were sacrificed by isofluran treatment (Abbott, Germany) on day 7 after induction of colitis. Colon samples from each mouse were removed under sterile conditions and collected in parallel for histopathological and immunohistochemical analyses as well as for detection of cytokines. For immunohistochemical stainings, colon samples were immediately fixed in 5% formalin and embedded in paraffin, and sections (5 μm) were stained with the respective antibodies as described below. Histopathology was investigated in paraffin-embedded hematoxylin and eosin (HE)-stained tissue sections of colon and lung. A published standardized histologic score ranging from

Fig. 1. Better clinical outcome of acute DSS colitis following (GPO)₁₀ treatment.

(a) Relative body weight loss, (b) clinical condition (as indicated by a clinical colitis score, see methods), and (c) relative colon length loss (in %) at day 7 following treatment with placebo (PLC; black bars), (GPO)₁₀ (GPO) application via the peroral (p.o.; gray bars) or intraperitoneal (i.p.; white bars) route from day 0 until day 7 were recorded in mice after DSS treatment for 7 days. Numbers of analyzed animals are given in parentheses. Mean values, standard errors of the mean (SEM), and significance levels as indicated were determined by the Student's *t*-test. Data are pooled from three independent experiments

0 to 6 was used for blinded evaluation of the inflammatory processes in the colon [24, 25].

Immunohistochemistry

In situ immunohistochemical analysis of colonic paraffin sections was performed as described previously [27, 28]. Primary antibodies against CD3 (#N1580, Dako, Denmark, dilution 1:10), B220 (eBioscience, San Diego, CA, USA, 1:200), myeloperoxidase-7 (MPO-7, # A0398, Dako, 1:10000), FOXP-3 (FJK-16s, eBioscience, 1:100), and Ki-67 (TEC3, Dako, 1:100) were used. For each animal, the average number of positively stained cells within at least six high power fields (HPF, $\times 400$ magnification) was determined by light microscopy.

Cytokine detection in colon culture supernatants

Colon biopsies were cut longitudinally, washed with PBS and strips of approximately 1 cm², as well as mesenteric lymphnodes (MLNs) and spleens were placed in 24-flat-bottom well culture plates (Nunc, Wiesbaden, Germany) containing 500 μ l serum-free RPMI 1640 medium supplemented with penicillin (100 U/ml) and streptomycin (100 μ g/ml; PAA Laboratories). After 18 h at 37 °C, culture supernatants were tested for IL-6, TNF- α , and IL-10 by the Mouse Inflammation Cytometric Bead Assay (CBA; BD Biosciences, Heidelberg, Germany) on a BD FACSCanto II flow cytometer (BD Biosciences) as described previously [25, 29, 30].

Statistical analysis

Medians, mean values, standard error of the means (SEM), and levels of significance were determined using appropriate tests as indicated (two-tailed Student's *t*-test). Two-sided probability (*P*) values ≤ 0.05 were considered significant. All experiments were repeated at least twice.

Results

Less intestinal immunopathology following (GPO)₁₀ treatment of mice with acute DSS colitis

Recent studies using mice lacking genes for MMP-2 and/or MMP-9 in experimental colitis models revealed that epithelial-derived MMP-9 is an important mediator in colitis induction whereas MMP-2 exerts protective function preserving intestinal epithelial barrier integrity [31, 32]. (GPO)₁₀ has been identified as a relevant binding structure for proMMP-2/-9 interfering with proMMP-2/-9 binding to the extracellular matrix. In our very recent *in vitro* study (published in the same issue), we could clearly demonstrate that, in cell lines, cellular proliferation and migration concomitant with matrix degradation could be stimulated due to MMP-(GPO)₁₀ interaction. Given that (GPO)₁₀ is capable of inducing MMP-2 activity *in vitro*, we were interested whether (GPO)₁₀ treatment could impact acute large intestinal inflammation *in vivo*. Mice were subjected to DSS in order to induce acute colitis (day 0) and treated with synthetic (GPO)₁₀ for 7 days either via the intraperitoneal (i.p.) or peroral (p.o.) route. At day 7

post induction (p.i.), i.p. (GPO)₁₀-treated mice had lost significantly less body weight as compared to placebo controls ($10.0 \pm 1.3\%$ versus $17.5 \pm 1.5\%$, $p < 0.005$; Fig. 1a). In addition, applying a “cumulative” clinical colitis score assessing body weight loss, occurrence of blood and consistency of stool revealed that mice benefitted from either (GPO)₁₀ treatment as compared to placebo controls suffering from severe colitis at day 7 p.i. (Fig. 1b). Given that colonic inflammation is accompanied by a significant shortening of the lower intestinal tract, we determined the lengths of the large intestines in treated and control animals. Mice treated with (GPO)₁₀ either p.o. or i.p. displayed significantly less colonic shortening ($21.4 \pm 1.5\%$ and $20.0 \pm 3.2\%$, respectively, $p < 0.05$) as compared to placebo mice ($31.2 \pm 3.1\%$; Fig. 1c). To further characterize the beneficial effects of (GPO)₁₀ during acute colonic inflammation, we studied the effects on colitis severity related histopathological changes in the colon. (GPO)₁₀ treatment via the i.p., but not p.o. route resulted in significantly less distinct colonic histopathology as compared to placebo controls at day 7 p.i. as indicated by lower histopathological scores in the i.p. (GPO)₁₀ group (Fig. 2). In i.p. (GPO)₁₀-treated animals, only few epithelial lesions and a discrete influx of inflammatory cells into the colonic lamina propria, but not into the submucosal layer, could be observed (mean histopathological score of approximately 3.0; Fig. 2). However, severe mucosal damage with extended ulcerations affecting the entire colon length and complete loss of the epithelium as well as a thickened submucosa with distinct transmural leukocyte infiltrates were present in the large intestine of the placebo group at day 7 p.i. (mean histopathological score of nearly 5.0; Fig. 2). Taken together, following i.p. (GPO)₁₀ treatment mice exhibited less macroscopic, clinical, and histopathological signs of large intestinal inflammation as well as less distinct inflammation-induced colon shortening following induction of acute DSS colitis.

Less distinct immune cell responses in colonic mucosa in situ following (GPO)₁₀ treatment of mice with acute DSS colitis

Given that human colitis is accompanied by the recruitment of pro-inflammatory immune cell populations to sites of inflammation in the large intestine [25], we next quantitated immune cell responses by immunohistochemical staining of colonic paraffin sections of animals with antibodies against CD3 (T-lymphocytes), B220 (B-lymphocytes), MPO7 (neutrophils), and FOXP3 (regulatory T-cells, Treg). In addition, colonic Ki67⁺ cells indicative for cell proliferation and regeneration were determined. At day 7, following colitis induction, placebo control mice displayed a substantial increase in T- and B-lymphocytes, neutrophils, and Tregs within their colonic mucosa as compared to naïve placebo mice (Fig. 3). The influx of CD3⁺, B220⁺, and MPO7⁺ cells, however, was significantly less pronounced in mice treated with (GPO)₁₀ by

Fig. 2. Less colonic histopathology of acute DSS colitis following (GPO)₁₀ treatment.

Histopathology scores of the colon were determined at day 7 following treatment with placebo (PLC; black bars), (GPO)₁₀ (GPO) application via the peroral (p.o.; gray bars) or intraperitoneal (i.p.; white bars) route from day 0 until day 7 in mice after DSS treatment for 7 days. Numbers of analyzed animals are given in parentheses. Mean values, standard errors of the mean (SEM), and significance levels as indicated were determined by the Student's *t*-test. Data are pooled from three independent experiments

either route at day 7 p.i. (Fig. 3a–c) whereas following i.p. (GPO)₁₀ application mice exhibited significantly higher FOXP3⁺ cell numbers ($p < 0.005$; Fig. 3d). Due to a relatively high standard deviation in the (GPO)₁₀ p.o. cohort only a trend towards higher colonic Tregs versus placebo controls at day 7 p.i. could be determined (n.s.; Fig. 3d). Interestingly, application of (GPO)₁₀ to naïve mice (i.e. without colitis induction) resulted in a 2- to 4-fold increase of neutrophils and Tregs, respectively, but not of T- and B-lymphocytes in the colonic mucosa *in situ* (Fig. 3). In addition, irrespective of the application mode, (GPO)₁₀-treated mice exhibited significant higher numbers of Ki67⁺ proliferative cells as compared to placebo mice with severe colitis (Fig. 3e). Taken together, less distinct clinical and histopathological signs of colonic inflammation following (GPO)₁₀ treatment were accompanied by lower numbers of pro-inflammatory immune cell populations such as T- and B-lymphocytes, but higher Treg and regenerative cell counts in the colonic mucosa *in situ*.

Intestinal and extra-intestinal cytokine secretion following (GPO)₁₀ treatment of mice with acute DSS colitis

To further underline the beneficial (i.e. immunomodulatory, anti-inflammatory) properties of synthetic (GPO)₁₀ treatment in acute DSS colitis, we next determined protein levels of the pro-inflammatory cytokine IL-6 in *ex vivo* colon cultures. Whereas during colitis induction,

Fig. 3. Quantification of defined cell populations in the colon of mice with acute DSS colitis *in situ* following (GPO)₁₀ treatment. The average number of cells positive for (a) CD3 (T-Lymphocytes), (b) B220 (B-Lymphocytes), (c) MPO7 (Neutrophils), (d) FOXP3 (regulatory T cells, Treg), and (e) Ki67 (proliferating cells) from at least six high power fields (HPF, $\times 400$ magnification) per animal was determined microscopically in immunostained colon sections isolated at day 7 following treatment with placebo (PLC; black bars), (GPO)₁₀ (GPO) application via the peroral (p.o.; gray bars) or intraperitoneal (i.p.; white bars) route from day 0 until day 7 in mice after DSS treatment for 7 days and compared to naïve (N) mice without colitis induction. Numbers of analyzed animals are given in parentheses. Mean values, standard deviations, and significance levels as indicated were determined by the Student's *t*-test. Data are pooled from three independent experiments

colonic IL-6 levels had increased multifold as compared to healthy mice, this increase, however, was significantly less pronounced following i.p. (GPO)₁₀ treatment as indicated by IL-6 concentrations reaching approximately 50% of levels in the placebo group at day 7 p.i. (Fig. 4). Thus, amelioration of colitis severity following i.p. (GPO)₁₀ application was paralleled by less IL-6 secretion from the inflamed colonic epithelium.

We were next interested whether (GPO)₁₀ treatment might also impact pro- and anti-inflammatory immune responses in extra-intestinal compartments such as the colon draining MLNs and the spleens. TNF- α levels increased more than 5-fold in MLNs derived from placebo control animals with severe colitis as compared to healthy mice (Fig. 5a). This effect was not as distinct following i.p. (GPO)₁₀ application as indicated by approximately 50% lower TNF- α levels in MLNs compared to placebo controls (Fig. 5a). In addition, i.p. (GPO)₁₀-treated mice exhibited significantly higher IL-10 levels in *ex vivo* MLN

and spleen biopsies as compared to placebo mice (Fig. 5b, c). Taken together, amelioration of acute DSS colitis following i.p. GPO treatment was paralleled by less distinct pro- and more pronounced anti-inflammatory cytokine responses in extra-intestinal compartments.

Pulmonary inflammatory responses following (GPO)₁₀ treatment of mice with acute DSS colitis

Given that intestinal inflammation might also be associated with extra-intestinal inflammatory responses, e.g. in the lungs as shown very recently by our group [33], we next examined HE-stained pulmonary paraffin sections of (GPO)₁₀ versus placebo-treated mice with acute colitis. At day 7, p.i. placebo mice with acute colitis displayed significant perivascular immune cell infiltrates in their lungs whereas these infiltrates were virtually missing in naïve as well as following p.o. and i.p. (GPO)₁₀ treatment (Fig. 6).

Thus, the presented data underline the beneficial anti-inflammatory effect exerted by (GPO)₁₀ treatment not only locally (i.e. at intestinal) but also at extra-intestinal tissue sites implicating therapeutic properties in acute intestinal inflammatory conditions in humans.

Discussion

Matrix metalloproteinases are essentially involved in mediating inflammatory responses in human IBD. So far, clinical studies investigating beneficial effects of synthetic compounds non-selectively blocking MMPs in cancer or inflammation were rather disappointing either due to lack of effectiveness or major negative side effects such as painful arthralgia as a consequence of collagenase blockage [34–38]. Thus, as potential pharmacological treatment options more effective and rather selective MMP blocking agents would be desirable.

The gelatinases A (MMP-2) and B (MMP-9) have been shown to be highly upregulated in intestinal inflammation in mice and men, *in vitro* and *in vivo* [11–16]. We have recently reported that selective blockage of gelatinases by the synthetic compound RO28-2653 was effectively ameliorating acute murine ileitis and colitis [39, 40]. Furthermore, studies in experimental colitis models revealed that MMP-2 exerts protective function preserving intestinal epithelial barrier integrity whereas tissue MMP-9 levels correlate with disease activity of patients with ulcerative colitis and have been associated with mucosal damage and fistulae in Crohn's disease. Further, MMP-9 is suggested to be important for neutrophil recruitment [31, 32, 41].

The synthetic collagen analogue (Gly-Pro-Hyp)₁₀, (GPO)₁₀, has been identified as a relevant binding structure for proMMP-2/-9. Given that (GPO)₁₀ interferes with collagen binding of proMMP-2 and proMMP-9 [20] and is

Fig. 4. Less colonic IL-6 secretion following (GPO)₁₀ treatment in acute DSS colitis.

IL-6 protein concentrations were determined in supernatants of colonic *ex vivo* cultures isolated at day 7 following treatment with placebo (PLC; black bars), (GPO)₁₀ (GPO) application via the peroral (p.o.; gray bars) or intraperitoneal (i.p.; white bars) route from day 0 until day 7 in mice after DSS treatment for 7 days and compared to naïve (N) mice without colitis induction. Numbers of analyzed animals are given in parentheses. Mean values, standard deviations, and significance levels as indicated were determined by the Student's *t*-test. Data are representative for three independent experiments

capable of inducing MMP-2 activity *in vitro* (as shown by our publication in the same issue), we were hypothesizing that (GPO)₁₀ might be a promising compound in pathophysiological conditions such as intestinal inflammation. Therefore, we investigated potential anti-inflammatory effects of (GPO)₁₀ in acute DSS colitis comparing peroral with intraperitoneal application mode. Our data revealed that intraperitoneal (GPO)₁₀ injection was more effective as compared to peroral application. This is not surprising

Fig. 5. Extra-intestinal cytokine secretion following (GPO)₁₀ treatment in acute DSS colitis.

Cytokine secretion was determined in supernatants of *ex vivo* biopsies taken from mesenteric lymph nodes (MLNs; (a) TNF-α, (b) IL-10) and (c) spleen (IL-10) isolated at day 7 following treatment with placebo (PLC; black bars), (GPO)₁₀ (GPO) application via the peroral (p.o.; gray bars) or intraperitoneal (i.p.; white bars) route from day 0 until day 7 in mice after DSS treatment for 7 days and compared to naïve (N) mice without colitis induction. Numbers of analyzed animals are given in parentheses. Mean values, standard deviations and significance levels as indicated were determined by the Student's *t*-test. Data are representative for three independent experiments

Fig. 6. Less pulmonary inflammatory responses following (GPO)₁₀ treatment in acute DSS colitis.

Paraffin sections of lungs taken at day 7 following treatment with placebo (left panel), (GPO)₁₀ (GPO) application via the peroral (middle panel) or intraperitoneal (right panel) route from day 0 until day 7 in mice after DSS treatment for 7 days (upper panel) were HE-stained and compared to naïve (N) mice without colitis induction (lower panel).

Representative photomicrographs for three independent experiments are shown ($\times 200$ magnification, scale bar 50 μm). Perivascular cuffs of infiltrating lymphocytes (arrows) are shown in lungs of placebo-treated mice with acute colitis (upper left), but missing in naïve mice or following (GPO)₁₀ treatment

given that (GPO)₁₀ is a polypeptide of 10 amino acid triplets which might have been substantially degraded during the gastroduodenal passage due to inactivating effects exerted by gastric and bile acids as well as pancreatic enzymes within the duodenal lumen.

Intraperitoneal (GPO)₁₀ treatment starting upon colitis induction resulted in significant better clinical and macroscopic outcomes as well as in significantly less histopathological changes of the colonic mucosa at day 7 as compared to placebo controls with severe colitis. Given that MMPs are involved in the recruitment of immune cells into the gut [18, 42] and shed biologically active IL-1, IL-6, and TNF- α molecules from the surfaces of effector cells such as neutrophils and macrophages, which, in turn, are able to induce MMP expression from immune, epithelial, and parenchymal cells [17, 18], it would be desirable to pharmacologically cut this vicious cycle all in one. Following i.p. (GPO)₁₀ treatment, amelioration of clinical symptoms and histopathology was paralleled by less infiltration of the colonic mucosa and lamina propria by immune and effector cells such as T- and B-lymphocytes, as well as neutrophilic granulocytes. This less distinct influx of immune cells into the large intestinal mucosa was accompanied by lower levels of pro-inflammatory cytokines such as IL-6 and TNF- α determined in the colonic mucosa of (GPO)₁₀-treated animals

as compared to placebo mice. The decrease in neutrophil numbers following (GPO)₁₀ application is well in line with previous reports by us [40, 43] and others [44, 45]: in acute experimental colitis, MMP blockage resulted in a diminished influx of neutrophilic granulocytes into the colon thereby reducing oxidative stress for the colon epithelium.

Importantly, at day 7, following colitis induction, numbers of FOXP3⁺ Tregs and Ki67⁺ regenerative cells were higher in the colon of (GPO)₁₀-treated mice as compared to placebo controls. Thus, (GPO)₁₀ exerts its beneficial effects *in vivo* by diminishing pro- and enhancing anti-inflammatory and thus promoting regenerative effects following intraperitoneal application in acute colitis. One explanation might be the interference of (GPO)₁₀ with the matrix binding of anti-inflammatory proMMP-2 and even more pronounced of pro-inflammatory proMMP-9. In contrast to proMMP-2, the effects of (GPO)₁₀ on proMMP-9 were not accompanied by an occurrence of proteolytic activity [20]. Another possible mode of action of (GPO)₁₀ might therefore be the release/removal of tissue sequestered pro-inflammatory proMMP-9 and concurrently the induction of MMP-2 with its protective function in preserving intestinal epithelial barrier integrity.

Recently, comparable results could be achieved by peroral treatment of mice suffering from acute DSS colitis with the selective gelatinase blocker RO28-2653 [40].

In this study, however, amelioration of acute colitis was accompanied by decreasing (and not increasing) FOXP3⁺ cell numbers in the colonic mucosa. Of note, in the study presented here, i.p. (GPO)₁₀ application resulted in increasing FOXP3⁺ Tregs in the colons not only of diseased, but also in healthy, naïve animals further underlying its potent anti-inflammatory capacity.

Surprisingly, the beneficial action exerted by (GPO)₁₀ was not restricted to the intestinal tract but also effective in extra-intestinal compartments: MLNs derived from i.p. (GPO)₁₀-treated mice secreted significantly less TNF- α as compared to placebo controls. Additionally, expression levels of the anti-inflammatory cytokine IL-10 were higher in MLNs as well as in spleens of i.p. (GPO)₁₀-treated mice versus placebo controls at day 7 p.i. Furthermore, whereas placebo mice displayed significant perivascular cuffs of inflammatory cells at day 7 p.i., the influx of inflammatory cells into lungs was virtually abolished following i.p. (GPO)₁₀ treatment. Thus, (GPO)₁₀ might be a promising pharmacological compound exerting its potent immunomodulatory (i.e. anti-inflammatory) effects locally in the intestinal tract as well as in extra-intestinal compartments.

Beside MMP-specific effects, (GPO)₁₀ might also impact immune-specific cellular processes directly, as it does via activation of immune receptors such as the platelet glycoprotein VI (GPVI) or the leukocyte-associated immunoglobulin-like receptor-1 (LAIR1) [26].

Given that to our knowledge (GPO)₁₀ is lacking non-selective MMP blocking, such as anti-MMP-1 and anti-MMP-7, properties the risk of significant unwanted side effects, such as arthralgia for instance, might be regarded as rather low [46]. Thus, from our current point of view, (GPO)₁₀ might be regarded a safe compound which needs to be further proven in future *in vivo* experimental and clinical studies.

Taken together, the data presented here demonstrate for the first time that the synthetic compound (GPO)₁₀ ameliorates acute murine DSS colitis. (GPO)₁₀ exerts its beneficial immuno-modulatory effect by disrupting the vicious cycle of the positive feedback loop between 'immune cell stimulation and MMP induction' and promotes anti-inflammatory and regenerative effects locally as well as systemically. Further mechanisms of (GPO)₁₀ action in experimental *in vivo* conditions by using MMP-2 and/or -9 deficient mice, however, need to be unraveled in ongoing studies. In conclusion, the beneficial features described here put (GPO)₁₀ in a promising position for future intervention strategies in human intestinal and extra-intestinal inflammatory diseases.

Acknowledgements

This work was supported by grants from the German Research Foundation (DFG) to S.B. and A.F. (SFB633, TP A7), A.A.K. and U.E. (SFB633, TP Z1), M.M.H. (SFB633, TP B6), R.S. (SFB366, TP C5 and C10; Ru624/3-2, Schu

646/1-10), and from the German Federal Ministry of Education and Research (BMBF) to S.B. ('Lab in a hanky' projects TP 1.1 and TP 8.2).

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

We thank Michaela Wattrödt, Ursula Rüschenhoff, Ger- not Reifenger, Uwe Lohmann, and the staff of the animal research facility for excellent technical assistance and animal breeding. We are grateful to Simone Spieckermann for immunohistochemistry staining of colon sections.

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