The Detergent Effect of Mesalazine Interferes with Phosphatidylcholine Binding to Mucin 2

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Keywords

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Abstract

Objectives: Therapeutically applied delayed-release phosphatidylcholine (PC) revealed mucosa protection and clinical improvement of ulcerative colitis. However, a recent trial with simultaneous application of delayed-release PC and mesalazine showed lack of efficacy. It is hypothesized that mesalazine acts as detergent to prohibit PC integration into mucus as target compartment, thus preventing topical mucus protection. **Methods:** In vitro PC-binding studies with mucin 2 and intestinally differentiated CaCo2 cells as well as outcome analysis of a therapeutic trial with delayed-release PC and additional mesalazine. **Results:** Choline-containing phospholipids, in particular PC, bind to mucin 2 as main scaffold protein of intestinal mucus to establish a hydrophobic barrier towards microbiota in the intestinal lumen. PC also binds to the apical surface of polarized CaCo2 cells with membrane-anchored mucin 3. Mesalazine removes mucin-bound PC and, thus, reduces transepithelial resistance. A post hoc analysis of patients from a previous multicenter

phase IIB trial with delayed-release PC revealed that those without mesalazine showed a PC dose-dependent outcome with regard to achievement of partial and complete remission ($p < 0.05$ for 1.6 and 3.2 g PC daily) whereas those treated simultaneously with mesalazine showed no PC dose dependency. **Conclusion:** Mesalazine solubilizes PC and, thus, prevents the protective action of therapeutically applied delayed-release PC within mucus.

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Introduction

Mesalazine is an established therapy for ulcerative colitis (UC) either as monotherapy in cases of mild to moderate disease activity or as an adjunct therapy in severe cases or for maintenance of remission [1, 2]. Its mode of action is not very well defined on a molecular level, but it is believed predominantly to be anti-inflammatory [3].

Due to its amphiphilic structure it may act as a detergent. It is applied to patients in a delayed-release preparation to exert its main action in the colon. The large intestine is not considered as absorptive organ for substrates with the exception of water and electrolytes. Therefore, it

is mainly the topical effect of mesalazine which mediates anti-inflammatory efficacy. However, a small portion of the applied aminosalicylates could also be absorbed in the small intestine. This part can cause adverse events such as kidney stones or in case of sulfasalazopyridine to be utilized for the treatment of rheumatoid arthritis [4–6].

The fact that mesalazine is a detergent may have implications on lipophilic substrates entering the colon. As recently described, there is a substantial amount of phosphatidylcholine (PC) secreted into the distal ileum for incorporation into the mucus to serve as a hydrophobic barrier towards the stool microbiota [7–9]. PC is believed to incorporate mainly into mucin 2 which is secreted by goblet cells and constitutes the main structural scaffold of the outer layer of the mucus [10]. After reabsorption of the bile acids in the terminal ileum, the outer mucus layer is believed to be more closely attracted to the colonic mucosa surface and moves distally to the rectum to constitute a dense protective hydrophobic shield towards the commensal flora [8].

In UC, the luminal secretion of PC is disturbed which mainly occurs in the distal ileum [7]. We have data which support a paracellular tight junction-mediated translocation process from systemic lipoproteins to the luminal side of mucosal cells driven by an electrochemical gradient involving cystic fibrosis transmembrane conductance regulator and binding to enterocyte apical plasma membrane-anchored mucin 3 from where it moves to mucin 2 in the upper mucus layer [11].

It is assumed that in UC the tight junctions are disrupted, and apical PC secretion is largely suppressed [11, 12].

Indeed, in UC the PC content of the mucus is intrinsically reduced by 70% compared to healthy controls or patients with Crohn's disease [13, 14]. Accordingly, the colonic mucosa is less hydrophobic and prone to microbiota-induced inflammation which always starts in the rectum, the last lawn of mucus PC content due to the longest exposure time to bacterial ectophospholipase activity in stool [15].

Accordingly, the concept arose to supplement the mucus PC content in patients with UC by application of a delayed-release oral PC preparation. This indeed proved to be effective in several trials [16–18]. Accordingly, a phase III trial was initiated with the primary end point of induction of remission. The trial included mesalazine-refractory UC patients [19]. For ethical and authority-suggested reasons, the patients had to maintain their mesalazine medication and had to take it simultaneously with the delayed-release PC preparation [19].

In this study the primary end point of achievement of remission was not met [20], despite the different preceding positive trials. Therefore, it was hypothesized that the simultaneous application of mesalazine as a detergent and the lipophilic PC, both in delayed-release preparations, may have had an impact on the topical bioavailability of PC for the colonic mucus.

It could be that mesalazine solubilizes PC which consequently stays as a micellar suspension in the lumen. Thus, it does not allow incorporation of PC into the mucus for hydrophobic protection, and therefore the aims of the present study are:

1. to show the specific binding property of PC to mucin 2;
2. the capability of mesalazine to remove PC from mucin 2;
3. the clinical consequences examined by a post hoc analysis of the clinical outcome of patients with UC in a multicenter phase IIB trial where PC was applied without mesalazine.

Materials and Methods

In vitro Lipid-Binding Studies to Mucin 2

Mucin 2 (M2378, Sigma) or – for comparison – fatty-acid-free bovine serum albumin (A6003, Sigma), both at 1 mg/mL phosphate-buffered saline (PBS, pH 7.4), were separately incubated in 1 mL with radiolabeled lipophilic or hydrophilic ligands at 10 mM in PBS. Lipids: [³H]PC, [¹⁴C]lysophosphatidylcholine, [³H]sphingomyelin, [¹⁴C]phosphatidylethanolamine, [³H]cholesterol, [³H]palmitate, and [³H]oleate. Hydrophilic ligands: [¹⁴C]inulin, [³H]sucrose, [³H]choline chloride, and [³H]taurocholate. All ligands were brought up with unlabeled ligand to 10 mM containing 100,000 cpm/mL. Radiolabeled compounds were purchased from Perkin Elmer (Waltham, MA, USA). Furthermore, the displacement of ligand binding was examined in the presence of increasing mesalazine (Y0000297, Sigma) or taurocholate (S0900000, Sigma) concentrations. This was followed by immunoprecipitation with respective antibodies from Santa Cruz Biotechnology for mucin 2 (B306.1/sc-59859) and for albumin (H126/sc-50535) at a dilution of 1:200 for 16 h [21]. After centrifugation and washing with PBS, the amount of precipitated ligands was determined.

PC-Binding Studies to the Apical Side of Polarized CaCo2 Cells

CaCo2 cells (ATCC®) were seeded in 12-well collagen-coated transwell culture dishes (0.4 µm pore size) at 7.5×10^4 cells/well (corresponding to 80–100 µg protein/well) and cultured in Dulbecco's modified Eagle's medium containing 5% fetal calf serum (Life Technologies, Carlsbad, CA, USA) for 3–30 days to allow apical/basolateral polarization and establishment of tight junctions, which was confirmed by measuring transepithelial resistance >400 Ω.

The PC binding to the apical surface of polarized CaCo2 cells was examined after incubation of 5–100 mM [³H]PC in 1 mL PBS for 1 h to determine the maximal binding capacity. At 100 mM

Fig. 1. Binding of lipophilic and hydrophilic ligands to mucin 2. Mucin 2 (1 mg/mL) was incubated with radiolabeled ligands (10 mM) in the absence or presence of 50 mM mesalazine or 50 mM taurocholate (TC). As ligand the phospholipids phosphatidylcholine (PC), lysophosphatidylcholine (LPC), sphingomyelin (SM) and phosphatidylethanolamine (PE) were employed as well as oth-

er lipophilic and hydrophilic substrates. After immunoprecipitation with anti-mucin 2 antibodies, ligand binding to mucin 2 was determined. **Inset** Concentration-dependent binding of PC to mucin 2. The maximal binding capacity for PC was 243 ± 49 nmol/mg mucin 2. Illustrated are means $\pm$ SD ($n = 6$); *** $p < 0.001$.

PC binding was quantified as a function of increasing (1–100 mM) mesalazine or taurocholate to determine IC_{50} . Removal of apical cell-bound [3H]PC was evaluated by exposure for 1 h with mucin 2 (1 mg/mL) in the presence of PBS alone or 50 mM mesalazine or taurocholate in PBS. The supernatant was immunoprecipitated with anti-mucin 2 antibodies, and [3H]PC was determined in supernatant and pellet. In addition, removal of cell surface-bound PC was accompanied by transepithelial resistance measurement.

Post Hoc Analysis of a Previous Phase IIB Trial with PC with Regard to the Presence or Absence of Mesalazine from the Study Population

This multicenter randomized, placebo-controlled trial of the modified release PC “LT-02” in mesalazine-refractory UC was approved by the central ethics committees of each participating country and complied with the guidelines for human studies including patient’s consent [18]. A total of 156 patients with inadequate response to mesalazine and a disease activity score (Simple Clinical Colitis Activity Index, SCCAI) of ≥ 5 and bloody diarrhea underwent treatment with 0.8, 1.6 or 3.2 g LT-02. The 3.2-g group improved by 51.7% from 8.5 to 4.1 SCCAI points ($p = 0.030$ in

comparison to placebo). Despite the obligation to maintain mesalazine therapy during the trial, 32 patients refused the intake of mesalazine but still remained in the trial population. In the present post hoc analysis, we evaluated the outcome of this rather small group of patients compared with those who followed the instructions and maintained a simultaneous application of LT-02 and mesalazine. Remission was defined as a SCCAI < 3 and partial remission as < 5 index points. Both groups were compared by the statistical analysis exactly described before [18]. A descriptive analysis of the data is presented in this study. For both groups we describe the PC dose-dependent response rates in the categories complete, partial and no remission.

Statistical Analysis

Statistical analysis was performed using Prism 4.0 software (GraphPad Software Inc., La Jolla, CA, USA). Differences between groups were evaluated according to the requested hypothesis using the Mann-Whitney U test. Multiple groups were compared by one-way ANOVA with a Dunnett’s post hoc test. Data are presented as means $\pm$ SD, $p < 0.05$ was considered statistically significant. For the post hoc analysis of the trial data, only an explorative p value was provided.

Fig. 2. Inhibition of PC binding to mucin 2 in the presence of increasing concentrations of mesalazine or taurocholate. Mucin 2 (1 mg/mL) was incubated with 100 mM [3 H]PC in the presence of increasing concentrations of mesalazine or taurocholate. Binding of [3 H]PC to mucin 2 was performed after immunoprecipitation with anti-mucin 2 antibodies. Illustrated are means $\pm$ SD ($n = 6$); * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to control.

Results

In vitro Lipid-Binding Studies to Mucin 2

First binding of various lipophilic and hydrophilic substrates to mucin 2 was examined after incubation of radiolabeled ligands (10 mM) with 1 mg/mL mucin 2 followed by immunoprecipitation. PC was the ligand with the highest mucin 2 binding capacity (Fig. 1). A dose-finding analysis revealed a maximal binding capacity of 243 ± 49 nmol PC/mg mucin 2 (Fig. 1, inset).

Due to a molecular weight of 154 kD for mucin 2, the amount of 1 mg corresponds to 6.5 nmol which would account for a PC-binding capacity of 37.4:1. In principle all three choline-containing phospholipids showed binding affinity to mucin 2 (PC, lysophosphatidylcholine and sphingomyelin). However, *in vivo* lysophosphatidylcholine is less abundant in mucus, and sphingomyelin does not pass to the mucus compartment [12]. Binding of choline-containing phospholipids was reversed by 50 mM mesalazine as illustrated in Figure 1. None of the other

tested lipophilic and hydrophilic compounds had affinity to mucin 2, and accordingly mesalazine had no effect. Moreover, we employed albumin as control protein which under physiological conditions is capable of binding lipophilic substrates (online suppl. Fig. 1; see www.karger.com/doi/10.1159/000493347 for all online suppl. material). Here no binding of choline-containing phospholipids was detectable after immunoprecipitation with anti-albumin antibodies. A background level of radioactivity was registered for all compounds and may reflect unspecific radioactivity trapping after immunoprecipitation, which was deducted from the registered total PC binding to mucin 2.

The mesalazine inhibition of PC binding to mucin 2 was dose dependent with an IC_{50} of 59 mM which was more efficient compared to taurocholate ($IC_{50} = 73$ mM) (Fig. 2). Physiologically the taurocholate-mediated solubilization of PC from mucin 2 operates as a luminal sink and helps *in vivo* for further recruitment of PC from systemic lipoproteins across tight junctions to the mucus

Fig. 3. Concentration-dependent binding of PC to the apical surface of polarized CaCo2 cells. The maximal surface binding capacity was 174 ± 25 nmol/mg protein. The transepithelial resistance (TER) across the polarized CaCo2 cell layer increased as a function of PC added, reaching a maximum of $501 \pm 84 \Omega$ at 100 mM PC added. Illustrated are means $\pm$ SD ($n = 6$).

Fig. 4. Inhibition of PC binding to the apical surface of polarized CaCo2 cells in the presence of increasing concentrations of mesalazine or taurocholate. The upper chamber of the transwell tissue culture chamber was filled with 50 mM $[^3\text{H}]$ PC in the presence of

increasing concentrations of mesalazine or taurocholate. Binding to the apical surface was measured as well as the transepithelial resistance (TER). Illustrated are means $\pm$ SD ($n = 6$); * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to control.

Fig. 5. Removal of PC (after incubation with 10 mM [3 H]PC for 1 h) from the apical surface of polarized CaCo2 cells by mucin 2 (1 mg/mL) in the presence of PBS, mesalazine (50 mM) or taurocholate (50 mM). Mucin 2 in PBS left PC bound to mucin 2, whereas mucin 2 in mesalazine or taurocholate left PC in the micellar solution, not bound to mucin 2. Mucin-2-mediated depletion of PC from the cell surface was in all cases associated with a drop in the transepithelial resistance (TER). Illustrated are means $\pm$ SD ($n = 6$); *** $p < 0.001$ compared to control.

compartment [7, 11, 12]. At the terminal ileum, when bile acids are quantitatively absorbed, the complete PC binds to mucin 2. This is not the case for mesalazine which stays in the colonic lumen.

PC Binding to Apical Plasma Membranes of Polarized CaCo2 Cells

The mesalazine effect was evaluated in polarized CaCo2 cells which contain the intrinsic, enterocyte apical membrane-anchored mucin 3 [11, 12]. Binding to the cell surface was saturated at 50 mM PC incubated (Fig. 3). PC binding to cells was reduced in the presence of mesalazine and taurocholate (Fig. 4). Moreover, when PC surface-loaded CaCo2 cells were equilibrated with mucin 2 (1 mg/mL) in the presence of PBS, mesalazine or taurocholate, almost the total amount of PC was removed in all cases. However, in the presence of mesalazine $67.7 \pm 8.1\%$ and in the presence of taurocholate $54.3 \pm 5.1\%$ remained in the aqueous phase, whereas with mucin 2 in PBS $82 \pm 11\%$ could be immunoprecipitated (Fig. 5; online suppl. Fig. 2). Increase and depletion of PC from the surface of polarized CaCo2 cells was associated with concomitant changes of the transepithelial resistance (Fig. 3–5).

Clinical Effect of the Presence or Absence of Mesalazine on Treatment Efficacy in UC Patients with a Modified-Release PC Preparation

In the previous phase IIB trial with delayed-release PC only the highest dose group with 3.2 g daily revealed an improvement of the SCCAI by 51.7% in comparison with 33.3% in the placebo group ($p = 0.030$). The remission rate in the 3.2-g group was 31.4% (11/35 patients) in comparison with 15% (6/40 patients) in the placebo group ($p = 0.089$) [18].

To highlight the protective role of PC when provided without mesalazine we performed now a post hoc subgroup analysis of the patients refusing the intake of mesalazine (32/156 patients) in comparison with the mesalazine group (124/156 patients). Patients with PC only achieved dose dependently higher rates of partial and complete remission compared with the mesalazine-PC group (Fig. 6; online suppl. Table 1). In the 3.2-g group 4 out of 8 patients and in the placebo group 1 out of 10 patients came into complete remission ($p = 0.0432$). In the mesalazine group no difference was registered among the various doses of applied PC (Fig. 6; online suppl. Table 1). However, in the placebo and 0.8-g LT-02 group, the outcome was better with mesalazine, which is provided always in the same dose. This could be due to its anti-inflammatory action.

Fig. 6. Clinical outcome after a 12-week treatment with placebo or increasing daily doses of delayed-release phosphatidylcholine LT-02 (LT) in a study population without and with concomitant mesalazine therapy. Illustrated are the percentages of patients in their respective group. The number of patients is marked in the columns.

Discussion

In this study it was shown that PC and the other choline-containing phospholipids lysophosphatidylcholine and sphingomyelin – but no other of the tested lipids – bind to mucin 2. PC maintains a hydrophobic mucus layer for mucosal protection, also represented by the transepithelial resistance. In contrast, the delayed-release formulation of mesalazine is capable of removing PC from mucin 2 and, thus, of impairing the protective lipophilic shield, represented by the loss of transepithelial resistance. This would indicate that mesalazine is a rather harmful therapy, because it may suppress the mucus PC content below a critical threshold. Indeed, deterioration of diarrhea is considered as a side effect of this therapy (see patient information sheet). On the other hand, the concomitant easier access to the mucosal surface may allow the anti-inflammatory action of mesalazine to be more effective. Indeed, mesalazine has an anti-inflammatory mode of action as shown in many trials. It is a symptomatic therapy with a rather low grade of efficacy, but low toxicity. Therefore, it is commonly chosen as the first-line therapy in mild to moderate UC. The empirically used therapeutic dose of 4 g daily mesalazine (26

mmol) could be responsible for keeping therapeutically provided delayed-release PC in a micellar solution within the intestinal lumen which prohibits its incorporation into mucin 2. This causes ineffectivity of the PC therapy, and the simultaneous application of PC and mesalazine is counterproductive. Potential impairing drug bioavailability may also in general be of concern, if other delayed-release medications are applied simultaneously.

Accordingly, the question of the present study was whether mesalazine could have an impact on the negative outcome of a recently conducted phase III induction of remission trial performed with a delayed-release PC preparation (LT-02) [19, 20]. In contrast to a series of previous clinical trials with positive results [16–18], the recent LT-02 induction of remission trial required for ethical consideration addition of mesalazine in all study populations. The reason behind this is that a placebo arm would leave patients with active inflammation without any proven therapy. The preceding phase IIB dose-finding trial with LT-02 identified a dose of 3.2 g PC daily as sufficient to achieve the primary end point of significant clinical improvement [16]. However, a retrospective analysis of the data revealed that a number of patients did not follow the required study guidelines and performed the study without taking the recommended dose of mesalazine. This small study population performed significantly better and achieved a dose-dependent partial or complete remission measured by the SCCAI (Fig. 6, left). On the other side, the remaining study population with simultaneous intake of mesalazine and PC showed no responsiveness to increasing PC doses (Fig. 6, right).

Critical View of the Study

The in vitro tissue culture experiments unequivocally showed that mesalazine impairs integration of PC into mucus, leading to a destabilization of its barrier function. For technical reasons the use of native ileal mucosal cells is not possible. Instead we had to work with the intestinal differentiated, polarized tissue culture system of CaCo2 cells originating from a colonic tumor cell line, which may not entirely match the physiological situation. However, the CaCo2 tissue culture setting has also been used by other research groups all over the world in numerous experiments to examine the mucosal barrier as well as absorption processes. An essential advantage of this cell line is their capability to establish a tight junction barrier.

To prove the therapeutic efficacy of PC either alone or together with mesalazine, we also analyzed the data of a

previous clinical trial. Although it is undoubtedly a shortcoming not to perform a new clinical trial on its own, we avoided hereby the obstacle to have a study arm without a proven therapeutic option since PC is not yet approved for this indication. The data originated from a prospective, randomized, placebo-controlled double-blind multicenter phase IIB trial where increasing doses of PC were provided.

A fraction of the study population refused to take mesalazine by their individual decision without being excluded from the trial. However, it is indeed a shortcoming that this mesalazine-free trial population consists of only 32 patients. Thus, the obtained exploratory statistical results showing a dose-dependent effect of PC have to be viewed with caution. At least they are suggestive to prove the concept that PC alone is effective and mesalazine should not be provided simultaneously. Thus, for future trials with PC it is advisable to avoid concomitant mesalazine application. Moreover, it would be interesting to evaluate the therapeutic efficacy for induction of remission in a head-to-head comparison between mesalazine and delayed-release PC preparations. In case confirmatory trials are successful, it would increase the armament of therapeutic options against UC by an inexpensive and harmless medication. PC would in addition target the pathophysiology of UC by strengthening the mucosal barrier and not simply act as a symptomatic anti-inflammatory drug.

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Statement of Ethics

This multicenter randomized, placebo-controlled trial of the modified release PC “LT-02” in mesalazine-refractory UC was approved by the central ethics committees of each participating country and complied with the guidelines for human studies including patient’s consent.

Disclosure Statement

The adult and financially independent children of W. Stremmel hold the patent for modified-release phosphatidylcholine and are shareholders of Lipid Therapeutics GmbH.

All other authors declare no conflicts of interest.

Author Contributions

W.S. designed the study and wrote the manuscript, S.S. performed the experiments and S.G. performed some statistical analyses.

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