Influence of Proton Pump Inhibitors and Histamine Receptor 2 Antagonists on *Blastocystis* ST3 and Selected Microorganisms of Intestinal Microbiota *In Vitro*

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**INTRODUCTION:** Proton pump inhibitors (PPIs) and histamine receptor 2 (H2) antagonists are commonly prescribed medications. Association between PPIs and alteration of the gut microbiota has been reported. *Blastocystis*, the most common intestinal protozoan worldwide, occurs in both healthy and symptomatic people with gastrointestinal or cutaneous disorders, with controversial pathogenicity. The current study was aimed to investigate the influence of PPIs and H2 blockers on the *in vitro* proliferation of selected intestinal bacteria, fungi, and protozoa.

**METHODS:** Cultures of *Lactobacillus rhamnosus*, *Escherichia coli*, *Enterococcus faecium*, *Candida albicans*, and *Blastocystis* subtype 3 were treated with different concentrations of respective medications *in vitro*, and the numbers of microorganisms were quantified and compared.

**RESULTS:** Pantoprazole and esomeprazole exerted a significant inhibition on *Blastocystis* and *C. albicans*, especially at higher concentrations, which were even more effective than metronidazole. On the other hand, treatment with pantoprazole caused an increase in proliferation of *L. rhamnosus* and *E. coli*. There was no influence of H2 blockers on the examined microorganisms.

**DISCUSSION:** PPIs, such as pantoprazole, can be a potential treatment in the prophylaxis or eradication of *Blastocystis* and *C. albicans*.

**ARTICLE**

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**INTRODUCTION**

Proton pump inhibitors (PPIs), such as pantoprazole, esomeprazole, and omeprazole, are commonly prescribed to treat a variety of medical conditions, including gastroesophageal reflux disease, gastric and duodenal ulcers, nonsteroidal anti-inflammatory drug–induced enteropathy, Zollinger–Ellison syndrome, dyspepsia, and *Helicobacter pylori* infection (1,2). PPIs are weak bases and can irreversibly inhibit the H+/K+ adenosine triphosphate pumps of parietal cells in the stomach lining, thus suppressing acid production and increasing the gastric pH, leading to changes in the composition of gut microbiota and parasitic colonization (3). As benzimidazole derivatives PPIs resemble benzimidazole 2-methylcarbamates (e.g., albendazole and mebendazole) in structure, and has been demonstrated to kill certain human protozoans *in vitro*, such as *Giardia lamblia*, *Entamoeba histolytica*, and *Trichomonas vaginalis* (4–6). Histamine type-2 receptor antagonists (H2 blockers), such as cimetidine and ranitidine, act by binding to type 2 histamine receptors on the basolateral surface of gastric parietal cells to interfere with the pathways of gastric acid production and secretion (7).

*Blastocystis*, a member of the *Heterokonta* or *Stramenopile* (8), is a genetically diverse unicellular parasite of unclear pathogenicity. It is one of the most commonly detected intestinal protists worldwide and found in both healthy and symptomatic people with gastrointestinal problems, such as diarrhea, abdominal pain, constipation, and flatulence (9,10). Association with skin disorders, including rush and urticaria, has also been reported (10–12), with controversial significance (13–15).

Many clinical observations indicate the influence of PPIs on the composition of gut microbiota (3,16,17), but the effect of H2 blockers is unknown. The actions and mechanisms of PPIs and H2 blockers on the diversity of gut microbiota, including the *Blastocystis* colonization, remain largely unclear. The current study was aimed to determine the *in vitro* sensitivity of selective gut microbiota to PPIs and H2 blockers in cell cultures.
Bacterial and fungal isolates and growth conditions

A lyophilized stock of the microorganisms was purchased in Micro Swabs form from the American Type Culture Collection (ATCC) via Merck (Warsaw, Poland). Isolates used in this study were the probiotic bacteria Lactobacillus rhamnosus (ATCC 7469) and Enterococcus faecium (ATCC 6057), gut commensal and opportunistic microorganisms Escherichia coli (ATCC 25922), and Candida albicans (ATCC 64548). Before start of the experiments, the bacterial and fungal isolates were freshly cultivated on Tryptone Soy Broth (TSB) (Merck, Warsaw, Poland) and Sabouraud broth, respectively. The bacteria were routinely subcultured on TSB (pH 7.2) every 2 days and incubated at 37°C, while the fungi were subcultured on Sabouraud broth (pH 5.9) every 6 days and incubated at 24.5°C. The microorganisms were all incubated under anaerobic conditions in tightly close polypropylene 12-mL Falcon tubes.

Bacteria and fungus preparation

Each bacterial isolate was harvested from TSB after 2 days of incubation by centrifugation at 5,525 g for 15 minutes and washed 3 times with sterile phosphate-buffered saline (PBS, pH 7.0). The pellet was suspended in sterile TSB, and the optical density (OD$^{600}$) of the bacterial suspension was adjusted to 1.5 ± 0.6 in TSB, with 1.19 × 10$^5$ colony-forming unit (CFU)/mL of E. coli, 1.22 × 10$^6$ CFU/mL of E. faecium, and 1.28 × 10$^6$ CFU/mL of L. rhamnosus. Aliquots of the bacterial suspension were diluted with PBS to 1:100, 1:1,000, and 1:10,000. From each dilution, 50 μL was spread on Tryptic Soy Agar plates (Merck) and incubated at 37°C for 2–4 days; then, the colonies were counted.

Candida albicans was harvested by centrifugation at 2,300g for 10 minutes, and washed 3 times in sterile PBS, then suspended in Sabouraud broth. The number of fungal cells was determined by counting in a Neubauer chamber (Heinz Herenz, Hamburg, Germany) and adjusted to 1.79 × 10$^6$ CFU/mL.

Treatment of the cultured gut microbiota with PPIs, H2 blockers, and metronidazole

Stock solutions of pantoprazole, esomeprazole, cimetidine, and ranitidine, with metronidazole as a reference antibacterial agent (19), were prepared by adding 10 mL of sterile distilled water to 20 mg of the drug to give a final concentration of 2 mg/mL. Since activation of pantoprazole is possible at pH 4, 2–3 drops of 1-mol HCl were added to lower the pH to simulate the conditions in the stomach. Just before the experiment, the pH of pantoprazole was adjusted to the output level (pH = 8.5) by adding 2–3 drops of 1-mol NaOH. Three concentrations, 0.1, 0.06, and 0.02 mg/mL, were prepared directly before use in the experiment (20,21). The final pH value of the solutions was 8.5, 5.8, 5.2, and 6.2 for pantoprazole, esomeprazole, both H2 blockers cimetidine and ranitidine, and metronidazole, respectively.

The number of Blastocystis ST3 was determined by counting them in a Neubauer chamber under ×400 magnification, with a final concentration in Jones’ medium at approximately 2.9 × 10$^6$ cells/mL. Treatment with different concentrations of drugs including metronidazole was performed in 5-mL tubes containing 4 mL of Jones’ medium and 1 mL of Blastocystis xenic culture, or 4 mL of TSB or Sabouraud broth and 1 mL of respective bacteria or fungi in triplicates. The same preparations without treatment were used as controls. The tubes were sealed and incubated at 37°C for 48 hours for bacteria, at 24.5°C for 6 days for Blastocystis, and at 37°C for 6 days for Blastocystis ST3 (20,21).

During the treatment, the number of Blastocystis cells was recounted and the pH value measured every day. The pH values were measured with laboratory pH meter inoLab Terminal 740 (WTW, Xylem Analytics, Germany). The viability of Blastocystis cells was assessed by staining with 0.4% Trypan blue solution, with the unstained cells being counted. The numbers of each bacteria and fungus cells were likewise assessed every 12 hours. The inhibition rates caused by the added agents were determined by the ratios of the microbial numbers between the treated groups and the untreated controls. All experiments were repeated 3 times, and the average values reported as results.

Statistical analysis

Significance in difference between the drug treatment and the controls was tested by the Student t test (GraphPad Prism 8). The Pearson χ$^2$ and 2-way analysis of variance test were used to compare the effectiveness between medications and the influence of the pH condition, respectively. Three-way analysis of variance...
(the Tukey test) was used to evaluate the influence of the drug concentrations adjusted to the incubation time. A P value of <0.05 was considered statistically significant.

**RESULTS**

**Pantoprazole was more effective than metronidazole in inhibition of Blastocystis ST3 in vitro**

Pantoprazole was more effective than esomeprazole or metronidazole in inhibiting the proliferation of Blastocystis at the concentrations of 0.1 mg/mL and 0.06 mg/mL, respectively (P < 0.0001), without difference in between (Figure 1). Esomeprazole and metronidazole showed no difference in the Blastocystis inhibition (P = 0.5628). The inhibitory effects of PPIs appeared from the third day of treatment and later, which was not seen with H2 blockers (ranitidine or cimetidine) (P = 0.7954 and P = 0.7802, respectively).

**Pantoprazole promoted proliferation of L. rhamnosus and E. coli in vitro**

The number of L. rhamnosus increased significantly after addition of 0.1- and 0.06-mg/mL pantoprazole from the first day of treatment (P < 0.0001), as compared to the control samples, in which the L. rhamnosus proliferation was observed at 12–48 hours (Figures 2 and 3). H2 blockers showed no significant influence (P = 0.0878). Neither PPIs (pantoprazole and esomeprazole) nor H2 blockers (ranitidine and cimetidine) had any influence on the proliferation of E. faecium (P = 0.2302, 0.5911, 0.3561, and 0.2449, respectively). The multiplication of E. coli was promoted by pantoprazole (P < 0.0001) (Figure 2), but not by esomeprazole, ranitidine, or cimetidine (P = 0.2595, P = 0.4850, and P = 0.8955, respectively) (Figure 3).

PPIs inhibited the proliferation of C. albicans

As compared to the controls, proliferation of C. albicans was inhibited by both PPIs tested in different concentrations from the third day of treatment (P = 0.005 for all the tests). There was no inhibition observed with H2 blockers. Metronidazole at the tested concentration did not inhibit the Candida proliferation.

Pantoprazole lowered the pH values in the cultures of Blastocystis, E. coli, E. faecium, and C. albicans

The results of pH values were the average of triple measurement (Table 1). Before treatment, the pH at incubation for 2 days was 6.0, 5.26, 4.96, and 6.3 for E. coli, E. faecium, L. rhamnosus, and Blastocystis ST3, respectively, while pH 4.79 for C. albicans at incubation for 6 days.

The pH value of Blastocystis treated with pantoprazole was 7.22 on the first day and 6.96 on the sixth day, which were higher than those of the controls with pH 6.3 on the first day and 6.54 on the last day of treatment (P < 0.0001). The pH values of Blastocystis treated with esomeprazole and H2 blockers did not change significantly, with 6.68 and 6.42 on the first day, while 6.54 and 6.66 on the sixth day, respectively.

The pH values of L. rhamnosus treated with pantoprazole, esomeprazole, H2 blockers, and controls were 7.01, 6.47, 6.2, and 6.75 on the first day, while 5.2, 5.28, 5.0, and 5.1 on the second day, respectively, without significant difference as compared to the conditions in the controls (P = 0.4303).

The pH values of E. coli/E. faecium cultures treated with pantoprazole, esomeprazole, and H2 blockers ranged at 7.3–7.05/7.09–6.92, 6.76–7.07/6.55–6.87, and 6.3–6.77/6.29–6.55, respectively, as compared to controls 6.96–6.44/6.80–5.38 on the
first and second day of treatment, respectively. Treatment with pantoprazole at 0.1 mg/mL caused significance increase in the pH values of the *E. coli* and *E. faecium* cultures as compared to controls (*P* = 0.0006 and *P* = 0.0002, respectively). Significant increase in pH values was observed in treatment of *E. faecium*, but not *E. coli* cultures, with esomeprazole, ranitidine, and cimetidine (*P* = 0.0015, *P* = 0.0081, and *P* = 0.0085, respectively).

Treatment of *C. albicans* with pantoprazole, esomeprazole, H2 blockers, and placebo showed pH values at 6.2, 5.66, 5.4, and 5.71 on the first day, and 5.35, 5.45, 5.26, and 5.13 on the sixth day, respectively, with statistical difference only seen with pantoprazole (*P* = 0.0039).

Incubation of the tested medications alone, without bacteria or fungi over the same period, did not show any changes in the pH values, indicating no degradation of the medications themselves in the culture medium.

**DISCUSSION**

The physiopathology of *Blastocystis* in human gut microbiota is incompletely understood. *Blastocystis* is usually considered as a common constituent of the healthy gut microbiota associated with higher bacterial diversity, while long-term asymptomatic carriage is not pathogenic (22,23). *Blastocystis* can act as an indicator for changes in gut microbiota (24), and Blastocystis colonization appears to link to eubiosis with a significantly higher *Faecalibacterium prausnitzii*-to- *Escherichia coli* ratio (25), in contrast to the gut dysbiosis observed in metabolic, infectious, or inflammatory diseases of the lower gastrointestinal tract (23).

However, some recent studies found that *Blastocystis* can suppress the beneficial gut bacteria, leading to a dysbiotic state (23). *Clostridiales* were significantly more abundant in *Blastocystis* colonized patients, whereas *Lactobacillales* more profuse in *Blastocystis*-free individuals (23). The amoebic form appears during optimal growth conditions of *Blastocystis* and may play a role in the exacerbation of intestinal symptoms (26). **In vitro** *Blastocystis* can adhere to intestinal epithelial cells and secrete cysteine proteases to contribute to pathogenesis (26). Correlation between elevated protease activity and a higher percentage of amoebic forms was demonstrated in isolates from the symptomatic patients (26). Such discrepant observations may be explained by the different subtypes and forms of *Blastocystis* with varying pathogenicity, and the diverse factors associated with alteration in the gut microbiota, including medications (27), as well as the dynamic interaction between *Blastocystis* and its cohabitants.

The pathogenesis of *Blastocystis* in gastrointestinal disorders remains debating (15,28,29), for the following reasons: (1) *Blastocystis* is detected in the stool samples of healthy people at prevalence rates of 36%–70%, with great regional difference (2,30–32). (2) Evidence for the pathogenic potential mainly comes from **in vitro** studies (3,33,34). (3) In comparison with other parasites, such as *Giardia, Cryptosporidium*, and *Entamoeba, Blastocystis* does not display morphologically virulent features such as flagella, although it secretes enzymes cysteine proteases and cathepsin B as putative virulence factors (4,33,34). Although the amoeboid form is usually detected in symptomatic individuals (35,36), no massive outbreaks associated with *Blastocystis* have been reported.

In view of the existing epidemiologic data (1,4), the current study demonstrated for the first time the inhibitory effect of PPIs
pantoprazole and esomeprazole on the proliferation of Blastocystis sp. in vitro. As compared to metronidazole, both pantoprazole and esomeprazole were found to exert significant influence on the different phyla of gut microbiota, encompassing bacteria, fungi, and protozoa. The antiprotozoal activity of PPIs has been demonstrated in vitro against Trichomonas vaginalis, Giardia intestinalis, and Entamoeba histolytica, with rabeprazole and pantoprazole being the most active compounds tested, even more potent than metronidazole (4). On the other hand, recent studies indicated association between PPI use and alteration of gut microbiota, with increased risk of infections, including Clostridium difficile (37). As compared to the nonusers, PPI users exhibited a significantly diminished abundance of gut commensals and lower microbial diversity, with increase in the riches of oral and upper gastrointestinal tract commensals, in particular Streptococcus, Staphylococcus, and Enterococcus, but a significant decrease in Faecalibacterium (3,38,39).

There is no consensus for an appropriate treatment of Blastocystis colonization, while well-controlled studies are scant. Some authors recommend treatment for those showing gastrointestinal or dermatologic disorders associated with significant parasite burden (>5 cysts per high-power field), but not the asymptomatic carriers with few cysts in the stool samples. Metronidazole is most widely used, with vastly inconsistent results (40,41). Other therapeutic options may include trimethoprim/sulfamethoxazole, nitazoxanide, paromomycin, tinidazole, and iodoquinol (42). Most of these medications have various significant side effects. It has been demonstrated that ingested probiotic

| Table 1. The pH changes during the treatment of different microorganisms with 0.1 mg/mL concentration of 4 medications—pantoprazole (PAN), esomeprazole (ESO), ranitidine (RAN), and cimetidine (CIM) |
|-----------------|----------------|----------------|----------------|----------------|
| Medication      | Microorganism  | Statistical analysis |
| Time of incubation (d) | Escherichia coli | | Enterococcus faecium | |
| PAN | ESO | RAN | CIM | Control |
| 0.5 | 7.30 | 6.76 | 6.50 | 6.50 | 6.96 | 0.0006a |
| 1 | 7.22 | 6.82 | 6.55 | 6.50 | 6.65 | 6.82 |
| 1.5 | 7.13 | 6.94 | 6.69 | 6.70 | 6.70 | 6.58 |
| 2 | 7.05 | 7.07 | 6.77 | 6.77 | 6.77 | 6.44 |
| 0.5 | 7.09 | 6.55 | 6.29 | 6.23 | 6.80 |
| 1 | 7.02 | 6.67 | 6.35 | 6.31 | 6.45 |
| 1.5 | 6.96 | 6.81 | 6.48 | 6.42 | 5.82 |
| 2 | 6.92 | 6.87 | 6.55 | 6.49 | 5.38 |
| 0.5 | 7.01 | 6.47 | 6.20 | 6.00 | 6.75 |
| 1 | 6.50 | 6.20 | 5.90 | 5.40 | 6.21 |
| 1.5 | 5.90 | 5.72 | 5.42 | 5.10 | 5.56 |
| 2 | 5.20 | 5.28 | 5.00 | 4.80 | 5.10 |
| 0.5 | 7.0 | 5.66 | 5.40 | 5.35 | 5.71 |
| 1 | 6.15 | 5.60 | 5.35 | 5.30 | 5.62 |
| 1.5 | 5.80 | 5.55 | 5.30 | 5.28 | 5.45 |
| 2 | 5.55 | 5.41 | 5.28 | 5.25 | 5.21 |
| 3 | 5.35 | 5.45 | 5.26 | 5.21 | 5.13 |
| 4 | 6.20 | 6.68 | 6.42 | 6.40 | 6.30 |
| 5 | 5.98 | 6.57 | 6.24 | 6.55 | 6.50 |
| 6 | 6.96 | 6.54 | 6.66 | 6.60 | 6.54 |

The value is presented as an average of 3 tested samples (P < 0.05).

aPAN according to the control sample.

bAll the tested medications according to the control sample.

cPAN according to the control sample.

dPAN according to the control sample.

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bacteria, such as *Lactobacillus* sp (43), or yeasts *Saccharomyces boulardii* (44), can inhibit the development of *Blastocystis* sp. In our previous study (45), a higher number of amoebic forms were observed in the first 2 days of coinubcation with *E. coli* and *E. faecium*, while in the next few days, *Blastocystis* proliferation was inhibited. The mechanisms of this contact inhibition remain to be determined.

In a successful eradication of *H. pylori*, a synergistic action of PPIs and antibiotics has been proposed (1). As *H. pylori* replicates more favorably at neutral pH, acid inhibition by PPIs can raise the pH *in situ*, meanwhile enhance the stability and activity of the antibiotics used, and in this way, increase the growth-dependent antibiotic efficacy (46). On the other hand, antibacterial properties of PPIs directly against *H. pylori* have been controversially observed *in vitro* (46,47). The antiprotozoal activity of PPIs has been demonstrated in a few *in vitro* and *in vivo* studies (1,4). PPIs were more effective than metronidazole in killing *T. vaginalis*, *G. lamblia*, and *E. histolytica* in cell cultures (4). Among the tested compounds, rabeprazole and pantoprazole were more active than omeprazole or lansoprazole, while pantoprazole was 134 times more effective than metronidazole in inhibition of *Blastocystis* and *C. albicans in vitro*. The mode of action may include direct antiproliferation and indirect regulation of the intestinal probiotic bacteria. Because of their high safety and tolerability, PPIs can be considered for clinical treatment of intestinal protozoan infections. Further studies are required to prove this concept and to establish the clinically ideal doses and regimens.

**CONFLICTS OF INTEREST**

Guarantor of the article: Ewa Dzika, PhD

Specific author contributions: Resources, M.L. and E.D.; conceptualization, M.L.; methodology, M.L. and W.C.; format analysis, M.L. and E.D.; investigation, M.L.; data collection and analysis, M.L. and C.I.; writing and draft, M.L.; review and editing, W.C. and C.I.; supervision, E.D.

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**Study Highlights**

**WHAT IS KNOWN**

- Epidemiologic data showed association between proton pump inhibitor use and gut microbiota.
- *In vitro* studies demonstrated inhibiting effects of proton pump inhibitors on *Helicobacter pylori* and certain parasites.
- Probiotic bacteria inhibited *Blastocystis* subtype 3 in cell cultures.

**WHAT IS NEW HERE**

- Pantoprazole and esomeprazole inhibited proliferation of *Blastocystis* subtype 3 and *C. albicans* in cell cultures.
- Pantoprazole enhanced *in vitro* proliferation of *L. rhamnosus* and *E. coli*.
- Cimetidine and ranitidine had no influence on the proliferation of bacteria, fungi, or protozoa.

**TRANSLATIONAL IMPACT**

- There is the clinical potential of proton pump inhibitors to regulate the homeostasis of gastrointestinal microbiota and to treat certain related infections.

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