SENSITIVITY OF *BLASTOCYSTIS* SP. CLINICAL STRAINS TO NEW BENZIMIDAZOLE DERIVATIVES

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Introduction

Blastocystis sp. (formerly *Blastocystis hominis*) is globally recognized as the most common protists of the intestinal tract of humans and many animal species [1, 2]. Over the past decades, significant progress has been made in studying the biological properties of these protozoa, the sources and mechanisms of human infection, the pathogenesis and clinical manifestations of *Blastocystis* sp. diseases (blastocystosis), and improving laboratory diagnostics and certain aspects of treatment of the latter [3-5].

At the same time, it should be noted that to date, there are no international or national guidelines for the treatment of blastocystosis, including approved medicines for this purpose (https://www.cdc.gov/parasites/blastocystis/health_professionals/index.html). Therefore, specialists from around the world use a wide range of drugs for the etiotropic treatment of blastocystosis: metronidazole, co-trimoxazole, nitazoxanide, paromomycin, iodoquinol, tinidazole, furazolidone, ketoconazole, fluconazole, ornidazole, norfloxacin, and others [3, 5-10].

Data on the clinical and parasitological efficacy of these drugs are controversial [3, 5, 7, 9-12]. Moreover, the results of recent clinical trials and *in vitro* experiments have demonstrated not only the low sensitivity of *Blastocystis* sp. strains to metronidazole, which has long been used as a first-line drug for the treatment of blastocystosis, but also the undesirable effect of this drug on stimulating survival mechanisms and increasing the virulence potential of parasites [13-16].

This has heightened the urgency of searching for new compounds with pronounced anti-Blastocystis activity to develop drugs based on them for the etiotropic therapy of blastocystosis.

In 2021, an international team of scientists studied the effect of two benzimidazole derivatives on the *Blastocystis* ST3 strain: pantoprazole and esomeprazole [17]. Both drugs belong to the group of proton pump inhibitors, are histamine receptor 2 (H2) antagonists and are used to treat gastric and duodenal ulcers, Zollinger-Ellison syndrome, reflux esophagitis, and Helicobacter pylori eradication (in combination with antibiotics).

The authors found that pantoprazole at concentrations 0.1 mg/mL and 0.06 mg/mL was more effective than esomeprazole or metronidazole in inhibiting the proliferation of *Blastocystis* ST3. The researchers concluded that certain benzimidazole derivatives could be a potential tool for the prevention or treatment of *Blastocystis* sp. infections.

The **aim** of the study was *in vitro* to determine sensitivity of clinical strains of *Blastocystis* sp. to new benzimidazole derivatives in comparison with metronidazole, as well as to investigate the effect of the subinhibitory concentration of the most promising benzimidazole derivative on the level of parasite virulence factors (formation of amoeboid forms and protease production).

Materials and Methods

Five clinical strains of *Blastocystis* sp. were used in the study, which were isolated from freshly collected fecal samples from patients with irritable bowel syndrome with predominant diarrhea (IBS-D, Rome IV). All fecal samples contained ≥ 5 parasite cells per field of view in wet smear preparations stained with 1% Lugol's solution under light microscopy with a total magnification of ×400. *Blastocystis* sp. was identified by microscopy of fecal smears permanently stained with Heidegger's iron hematoxylin and trichrome as modified by Wheatley [18] (Fig. 1).

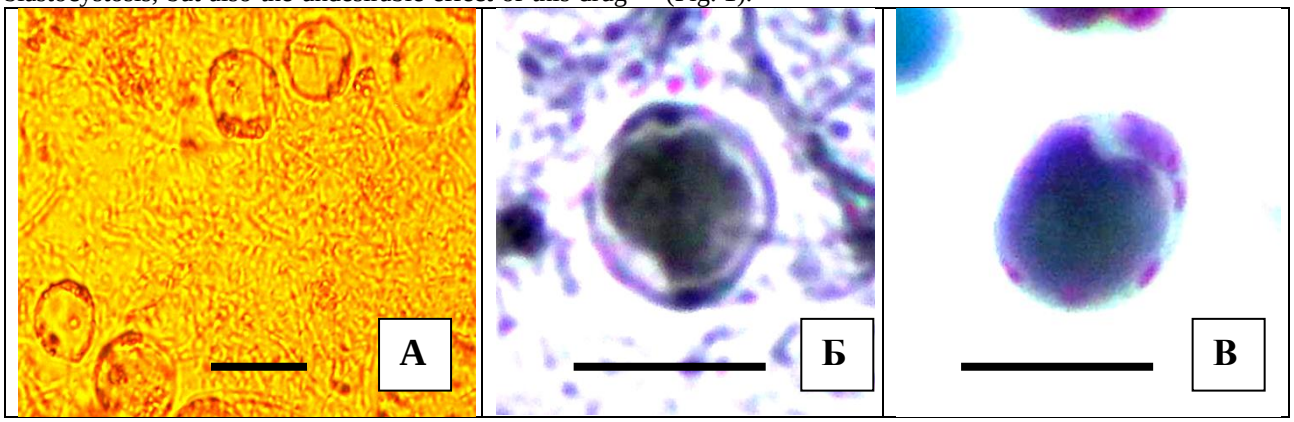

Fig. 1. *Blastocystis* sp. in fecal smear preparations of patients with IBD-D stained with 1% Lugol's solution (A), Heidenhain's iron hematoxylin (B) and trichrome to Wheatley's modification (C) (light microscopy, reference mark 10 µm).

Cultures of *Blastocystis* sp. were grown at 37 °C under anaerobic conditions in RPMI-1640 liquid nutrient medium with added antibiotics and inactivated horse serum as described previously [19]. Stabilized (long-term) xenic cultures of parasites were obtained from primary cultures after ten consecutive subcultures in a new portion of the

DOI: 10.5281/zenodo.8046133

medium. The sensitivity of *Blastocystis* sp. to new benzimidazole derivatives, metronidazole and various ethanol concentrations was determined at an initial concentration of parasite cells in suspensions of 2×10^5 / ml.

A group of new benzimidazole derivatives (NBDs) included: tris-benzimidazole aconitic acid (BAA), benzimidazole histidine (BHI), benzimidazole lipoic acid (BLA) and bis-benzimidazole lysine succinamide (BBL), which were first synthesized and provided (in the form of solutions with a concentration of each compound of 10 mg/ml) by the head of the laboratory and clinical department of molecular immunopharmacology of the Mechnikov Institute of Microbiology and Immunology, Professor A. Martynov. Structural chemical formulas of BAA, BPI, BLΦ and BBL are shown in Fig. 2.

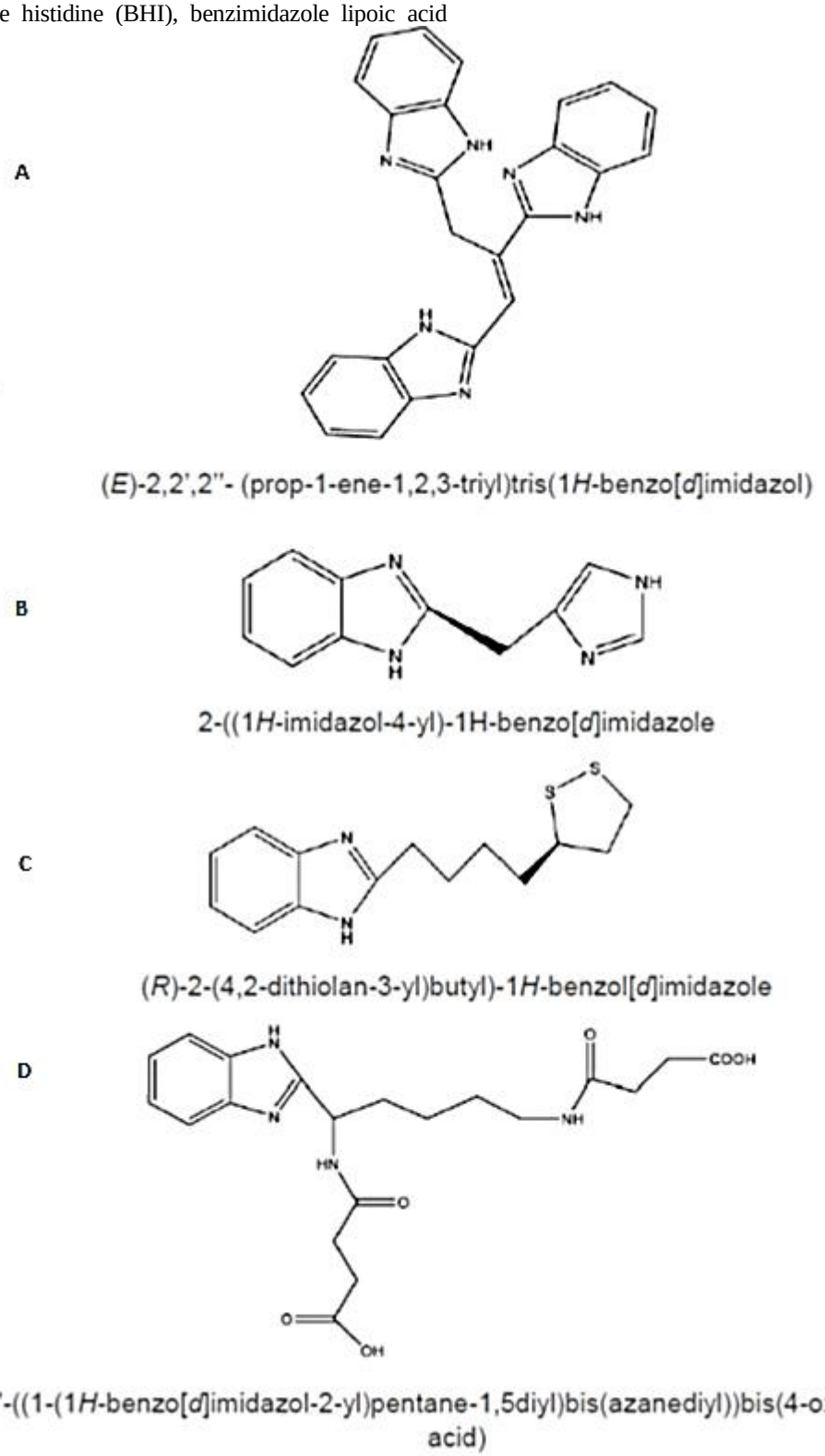

Fig. 2. Structural chemical formulas of new benzimidazole derivatives: BAA (A), BHI (B), BLA (C) and BBL (D).

DOI: 10.5281/zenodo.8046133

The comparison drug was used as a solution for infusion "Metronidazole-Darnitsa" with 5 mg/ml metronidazole (MTZ) (PrJSC "Pharmaceutical Firm "Darnitsa", Ukraine).

When studying the anti-*Blastocystis* activity, NBDs and metronidazole were tested in the range from 1000 μg/ml to 1 μg/ml (with a twofold decrease in their successive concentrations). Each series of experiments included control tubes for parallel evaluation of the growth rate of *Blastocystis* sp. in RPMI medium without any antiparasitic agents added and with 96 % ethanol added to the parasite cultures to final concentrations of 10.0 %, 5.0 %, 2.5 % and 1.0 % (w/v) to assess the role of ethanol in the effects of the corresponding concentrations of BAA and BLA on *Blastocystis* sp.

The presence and number of viable *Blastocystis* sp. cells in all tubes were determined daily for four days (24, 48, 72, and 96 h) due to the beginning of a natural decrease in the concentration of parasites when they were grown in RPMI medium [19]. *Blastocystis* sp. cells were counted in a hemocytometer using the trypan blue dye exclusion test, which was reproduced according to the basic protocol [20] with the difference that the cells were washed from the medium's serum by centrifugation at 500 g for 5 min.

The following cell counting techniques of *Blastocystis* sp. and criteria for assessing their viability: each counting procedure was performed in parallel in two hemocytometers by two different specialists under light microscopy with a total magnification of ×100; cells stained in blue were considered non-viable, and non-stained (intact) cells were considered viable; Cells with an indeterminate status according to the staining criterion were subjected to microscopy at ×400 magnification to detect signs of destruction (destruction of the cell wall and internal elements), cells without signs of destruction were considered viable.

The index of inhibition of growth (reproduction) of *Blastocystis* sp. cells due to the effect of different concentrations of NBD, MTZ and ethanol on parasite cultures was calculated daily using the formula: GI% = (A - B)/A×100, where GI% is the level of inhibition of *Blastocystis* sp. growth in percentage (%), A is the average number of viable parasite cells in the adequate control tube, B is the average number of viable cells in the experimental tube with parasite culture to which NBD, MTZ or ethanol were added. For the calculation of GI% of MTZ, BHI, BBL and ethanol, tubes with *Blastocystis* sp. cultures in RPMI medium without any antiparasitic agents added (hereinafter referred to as untreated control) served as adequate control, and for GI% of BAA and BLA, tubes with parasite cultures grown in the presence of appropriate concentrations of ethanol were used as control tubes to take into account its role in the manifestation of the anti-*Blastocystis* effect of these agents.

The anti-*Blastocystis* activity of all NBDs and MTZ was characterized by the following indicators: the minimum (lowest) inhibitory concentration of the drug that inhibits the growth of all parasite cultures by 50% (MIC_{50}); the minimum lethal concentration that completely (100%) destroys the cells of all parasite strains (MLC). MIC_{50} is the arithmetic mean with the mean linear deviation (M±m) of the GI% function, which was calculated using the two closest (smaller and larger than 50% inhibition) empirical GI% values according to the formula: $MIC_{50\%}$ = (test agent concentration × 50)/GI% empirical.

The MLC indicator was determined by the presence/absence of mildew in those four-day-old cultures in which no *Blastocystis* sp. cells with clear signs of viability were microscopically detected. For this purpose, 1.0 ml of the test crop was taken, washed twice from drug residues by centrifugation in RPMI medium (volume ratio 1:9; centrifugation mode 500g, 5 minutes), the resulting precipitate was resuspended in 0.3 ml of fresh RPMI and incubated for four days as described earlier.

Morphological changes of *Blastocystis* sp. cells due to the lethal effect of NBDs were described by the results of their phase-contrast microscopy at a total magnification of ×600. The effect of subinhibitory concentrations of MTZ and the most promising NBD on the formation of amoeboid forms of *Blastocystis* sp. and on the level of protease activity of parasite cell lysates was determined according to the protocols described in detail in a previous publication [21].

All experiments were performed in triplicate. Statistical data processing was performed using IBM SPSS Statistics v.19.0 software. Differences in mean values (M±m) were considered statistically significant at $p < 0.05$.

Results and discussion

The potential suitability of certain benzimidazole derivatives for the development of etiotropic therapy of intestinal invasions caused by *Blastocystis* sp. has recently been demonstrated by an international group of scientists (Poland, Germany, Taiwan) in [17]. This article presents for the first time the results of an in vitro study of the sensitivity of 5 clinical strains of *Blastocystis* sp. to 4 NBDs (BAA, BHI, BLA and BBL) in comparison with the anti-*Blastocystis* activity of MTZ, as well as the priority results of the effect of the subinhibitory concentration of the most promising NBD on the level of parasite virulence factors (formation of amoeboid forms and protease production).

In the untreated control of one-day *Blastocystis* sp. cultures, the concentration of viable parasite cells was $(2.8 \pm 0.5) \times 10^5$ / ml, and in the subsequent incubation period, an exponential increase in their number was observed with a maximum concentration of $(56.6 \pm 9.0) \times 10^5$ cells/ml in three-day cultures (hereinafter referred to as the stationary growth phase). The relative number of viable *Blastocystis* sp. cells in their one-day cultures reached (98 ± 2) %, and on the fourth day of incubation decreased to (81 ± 7) %.

The inhibitory effect of ethanol on the *Blastocystis* sp. growth directly depended on its concentration in the medium and was most pronounced after the first day of incubation of parasite cultures and gradually decreased by the end of the observation period, which is probably due to a regressive decrease in the initial amount of ethanol. In one-day and four-day cultures of *Blastocystis* sp., the GI% of ethanol was, respectively: (32 ± 5) % and (19 ± 4) % at an initial ethanol concentration of 10 %; (18 ± 4) % and (5 ± 1) % at an initial ethanol

DOI: 10.5281/zenodo.8046133

concentration of 5 %; (4 ± 1) % and 0 % at an initial ethanol concentration of 2.5 %; at 1 % ethanol in the medium, the effect of inhibiting the growth of *Blastocystis* sp. was not observed.

In general, the anti-*Blastocystis* activity of BAA, BHI and BLA (as well as that of the comparison drug MTZ), which we have established, is characterized by a direct positive pattern in the concentration-response and contact time-response effects. That is, both the increase in the concentration of these benzimidazole derivatives and the time of their action on *Blastocystis* sp. cultures clearly increase the manifestations of growth inhibition and parasite cell dehydration (Table). Instead, it was found that the effect of different concentrations of BBL on *Blastocystis* sp. strains, in the context of the "concentration-response" effect, is variable - with different directions of manifestation of this effect.

In the concentration range from 1000 μg/ml to 64 μg/ml, BBL has a certain inhibitory effect on the growth of *Blastocystis* sp., and in the concentration range ≤ 32 μg/ml it clearly stimulates the reproduction of parasite cells in crops. The greatest stimulation of growth of *Blastocystis* sp. cultures was observed at a concentration of BBL in the medium of 16 μg/ml, which provided an increase in the number of parasite cells by about 1.8 times in the stationary growth phase compared to the untreated control ($p < 0.05$). In addition, among the studied cultures of *Blastocystis* sp. a relatively more pronounced variation in sensitivity to BBL than to BAA, BHI and BLA was observed.

In general, the analysis of the actual MIC_{50} and MLC values using the rank U-test showed the following ranking gradation of NBDs in the sequence from the highest to the lowest level of anti-*Blastocystis* activity (Table): BLA, BAA, BHI and BBL (*U*f < *U*st).

Table - Anti-Blastocystis activity of BAA, BHI, BLA, BBL and MTZ in terms of MIC_{50} and MLC

Active ingredient	MIC_{50} (M ± m, μg/ml)				MLC (μg/ml)			
	24 h	48 h	72 h	96 h	24 h	48 h	72 h	96 h
BAA	16,6 ± 8,5	12,7 ± 2,8	10,9 ± 2,8	7,0 ± 2,1	1000	250	125	64
BHI	20,6 ± 7,6	15,2 ± 6,8	11,7 ± 3,0	5,9 ± 1,8	-*	500	250	125
BLA	13,5 ± 5,5	10,3 ± 2,1	7,2 ± 2,6	4,1 ± 1,0	1000	250	125	32
BBL	264,8 ± 51,3	189,9 ± 37,5	155,0 ± 31,4	102,5 ± 16,2	-*	-*	-*	-*
MTZ	20,1 ± 8,1	14,3 ± 2,6	12,5 ± 3,2	6,4 ± 1,8	-*	1000	500	250

*Notes. *- the used concentrations of BHI, BBL and MTZ did not provide the effect of complete killing of Blastocystis sp. cells*

In the stationary growth phase of *Blastocystis* sp. cultures, the MIC_{50} value for BLA was (7.2 ± 2.6) μg/ml and was 1.5, 1.6 and 21.5 times lower than the values of this indicator for BAA, BHI and BBL, respectively ($p < 0.05$). In addition, BLA showed a higher inhibitory effect (GI%) on clinical strains of *Blastocystis* sp. compared to MTZ: in the stationary phase of growth of treated parasite cultures, the established MIC_{50} values for BLA were 1.7 times lower than those for MTZ ($p < 0.05$). On the other hand, the growth inhibition rates of parasite cultures treated with BAA, BHI and MTZ are similar: with an insignificant difference in their MIC_{50} ranging from 4.9% to 13.8% ($p > 0.05$). And BBL is significantly inferior to MTZ in terms of the effectiveness of inhibiting the growth of *Blastocystis* sp. In the phase of stationary growth of parasite cultures, the MIC_{50} of BBL is 12.4 times higher than the MIC_{50} of MTZ.

Thus, among a studied NBDs, the BLA is characterized by the relatively highest efficiency of inhibition of *Blastocystis* sp. growth *in vitro*, exceeding MTZ by 1.7 times ($p < 0.05$).

According to our experiments, the effect of lethal action on *Blastocystis* sp. cells is most pronounced in BLA and BAA (Table). Only exposure to these NBDs at the highest concentration used (1000 μg/ml) resulted in complete killing of all clinical strains of *Blastocystis* sp. during the first day of incubation. In the period corresponding to the phase of stationary growth of parasite cultures in the untreated control, the actual MLC value (125 μg/ml) of both BLA and BAA was 2 and 4 times lower, respectively, than the MLC values established for BHI (250 μg/ml) and MTZ (500 μg/ml) ($p < 0.05$). In contrast, it was not possible to determine the MLC of BBL - even the highest concentrations of BBL during the entire four-day period of cultivation of *Blastocystis* sp. did not lead to the killing of their cells.

Currently, the mechanism of inhibitory/lethal effect of benzimidazole derivatives on *Blastocystis* sp. remains unclear [17]. Using the phase-contrast microscopy method, we found a deep destruction of *Blastocystis* sp. cells that occurs when parasite cultures are exposed to lethal concentrations of BLA: the central body disappears in the cells, intensive formation of granules and vacuoles, thinning and loss of a clear outline of the outer membrane, which finally collapses completely, and detritus of their internal contents is found in place of the cells (Fig. 3).

DOI: 10.5281/zenodo.8046133

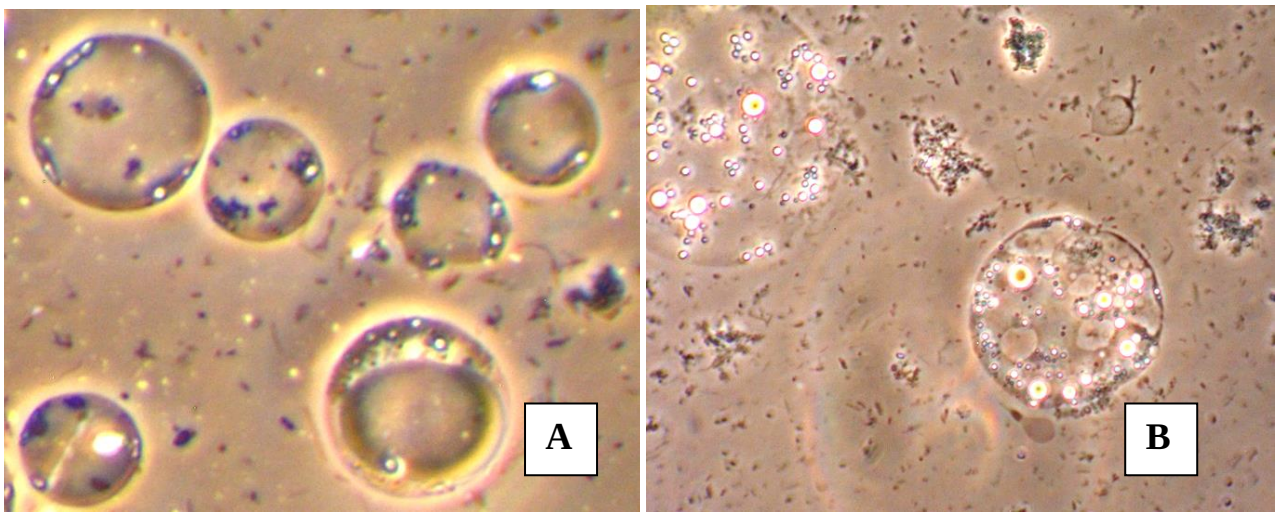

Fig. 3 Morphological changes in *Blastocystis* sp. cells resulting from the lethal effect of BLA: intact parasite cells (A); parasite cells with signs of deep destruction caused by the action of lethal concentrations of BLA (B).

The results of our study of the clinical strains of *Blastocystis* sp. sensitivity to NBDs are generally consistent with the data and the essence of the conclusions of foreign scientists [17]. Briefly, first, certain benzimidazole derivatives have antiparasitic effects against the blastocystosis pathogen; second, the level of anti-*Blastocystis* activity varies significantly among different compounds of this group (with different structural chemical formulas); third, some benzimidazole derivatives are significantly superior to MTZ in terms of the effectiveness of inhibiting the growth of *Blastocystis* sp. cultures in vitro.

In the context of the latter provision, the authors of the article [17] recognized pantoprazole as an effective drug, which, in their opinion, can be additionally included in the pharmacotherapeutic group of antiparasitic drugs. In addition, they noted the high safety and tolerability of pantoprazole by patients, which substantiates the prospects of its clinical use for the treatment of intestinal protozoan infections. Unfortunately, the authors of the study [17] did not determine the levels of inhibitory and lethal effects of pantoprazole on *Blastocystis* sp. strains by MIC (MIC_{50}, MIC_{90}, or other) and MLC, which makes it impossible to adequately compare their data with the values of the corresponding indicators established by us for NBDs (primarily for BLA).

At the same time, foreign scientists rightly emphasize the need for further research to confirm the clinical (*in vivo*) efficacy of benzimidazole derivatives, study the effect of the latter on various intestinal microbiota, and establish "ideal" doses and regimens for the treatment of patients with blastocystosis and other protozoal diseases. However, in our opinion, an equally important scientific task is to determine the nature and extent of the effect of relatively low concentrations of benzimidazole derivatives on the virulent potential of the blastocystosis pathogen (intensity of formation of amoeboid forms and protease production).

It is known that amoeboid forms are a stage of the life cycle of *Blastocystis* sp. and play a pathophysiological role in the onset and course of blastocystosis, so their formation is a sign of virulent strains of parasites [3, 7, 14, 21, 22]. Work [19] shows that when *Blastocystis* sp. is grown in RPMI medium, amoeboid forms reach their peak number on the fourth day of incubation (post-stationary growth phase). During this period, the relative number (percentage) of amoeboid forms (PAF) in the untreated control of the *Blastocystis* sp. strains we studied varied from 9.1 % to 14.9 % with an average value of (12.0 ± 2.9) %. At all concentrations of MTZ, which did not provide its lethal effect on *Blastocystis* sp. cells, the presence of amoeboid forms was observed in parasite cultures.

A decrease in the concentration of MTZ to ≥ 64 μg/ml was accompanied by a marked increase in PAF. At a subinhibitory concentration of the drug of 2 μg/ml, PAF was almost 2.5 times higher than in the untreated control ($p < 0.05$). Moreover, under the conditions of MTZ concentration ≥ 4 μg/ml, an increase in the total number of parasite cells was observed in *Blastocystis* sp. cultures compared to the untreated control, with the greatest increase (1.4 times) detected in crops with a subinhibitory concentration of 2 μg/ml.

According to the results of our studies, subinhibitory concentrations of BLA (≤ 2 μg/ml) did not stimulate the proliferation of *Blastocystis* sp. cells, but caused an increase in PAF. In the crops treated with low concentrations of BLA, abemoid cells of *Blastocystis* sp. were detected after the first day of incubation, in the subsequent periods, the PAF gradually increased and reached peak values in the post-stationary phase of parasite growth.

In four-day cultures of different strains of *Blastocystis* sp. with a concentration of BLA of 2 μg/ml, the PAF varied from 14.5% to 23.8% with an overall average value of (19.2 ± 4.6) %, i.e., it was 1.6 times higher than its value in the untreated control ($p < 0.05$). Thus, the effect of subinhibitory concentrations of BLA on the growth of *Blastocystis* sp. *in vitro* increases the formation of amoeboid forms of parasites by 1.6 times compared to the untreated control, but this effect is as much less than similar subinhibitory concentrations of MTZ ($p < 0.05$).

DOI: 10.5281/zenodo.8046133

The authors of publications [21, 22] found that strains of *Blastocystis* sp. that produce a higher PAF are characterized by increased protease activity (PA). The correlation coefficient between the actual values of these properties in parasite strains isolated from symptomatic individuals reached +(0.95-0.96). However, in another study [14], under the influence of a subinhibitory concentration of MTZ (1 μg/ml), researchers noted a certain increase in PA by both *Blastocystis* sp. isolates that formed amoeboid forms and those isolates in which these forms were not detected.

We studied the effect of subinhibitory concentrations of BLA (2 μg/mL) and MTZ (2 μg/mL) on the PA level of clinical strains of *Blastocystis* sp. isolated from patients with IBS-D, the comparative results of these experiments are shown in Fig. 4.

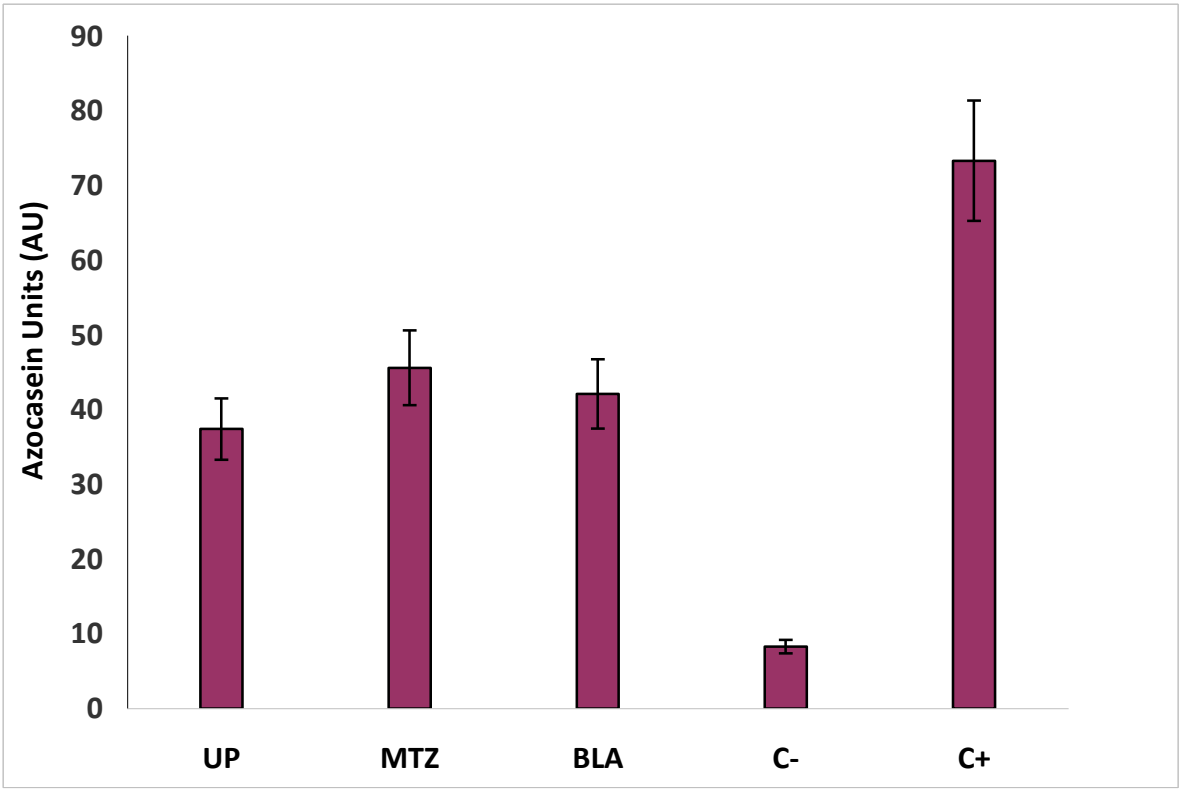

Fig. 4 Effect of subinhibitory concentrations of BLA and MTZ on the level of protease activity of *Blastocystis* sp.

Notes: one unit of azocasein (AU) is the number of proteases with enzymatic activity that causes an increase in the measured optical density (OD) of azocasein hydrolysis products by 0.01 units per hour; UP - lysates of *Blastocystis* sp, obtained from untreated parasite cultures; MTZ - lysates of *Blastocystis* sp. cells obtained from parasite cultures treated with a subinhibitory concentration of MTZ (2 μg/ml); BLA - lysates of *Blastocystis* sp. cells obtained from parasite cultures treated with a subinhibitory concentration of BLA (2 μg/ml); C- - negative control, in which the azocasein cleavage reaction was performed with thermally inactivated (at t = 90 oC for 20 min) lysates of *Blastocystis* sp, cell lysates obtained from untreated parasite cultures; C+ - positive control, in which the azocasein cleavage reaction was performed with a working solution of trypsin (2.5 mg/ml trypsin in phosphate-salt buffer with pH = 7.4).

It was found that under the influence of a subinhibitory concentration of MTZ (2 μg/ml), PA of *Blastocystis* sp. cell lysates increased by 1.2 times compared to the untreated control ($p < 0.05$). The effect of a subinhibitory concentration of BLA (2 μg/ml) also increases the value of PA of parasite cell lysates to some extent. This increase actually amounts to 8.2 AU, i.e., 11.2 % of the PA level of *Blastocystis* sp. cell lysates grown in the untreated control ($p > 0.05$).

The difference between the values of PA of parasite cell lysates obtained from cultures treated with identical subinhibitory concentrations of BLA and MTZ does not reach statistical significance: the former is only 3.5 AU lower than the latter, which is 7.7 % ($p > 0.05$). Thus, the effect of subinhibitory concentrations of BLA to some extent increases the PA of *Blastocystis* sp. cells, but according to this criterion of influence on the virulent potential of parasites, BLA at least does not exceed the effect of subinhibitory concentrations of MTZ.

Conclusions

1. According to the results of *in vitro* susceptibility study of 5 clinical strains of *Blastocystis* sp. to the action of 4 new benzimidazole derivatives (BAA, BHI, BLA and BBL) and metronidazole (MTZ), it was found that the anti-*Blastocystis* activity of BAA, BHI, BLA and MTZ reflects a direct positive pattern in the concentration-response and contact time-response effects, while different concentrations of BBL have different directions of action on *Blastocystis* sp.

2. In the phase of stationary growth of *Blastocystis* sp. cultures, the MIC_{50} value for BLA was (7.2 ± 2.6) μg/ml

DOI: 10.5281/zenodo.8046133

and was 1.5, 1.6, 21.5 and 1.7 times lower than that for BAA, BHI, BBL and MTZ, respectively ($p < 0.05$). In addition, BLA is 4 times more effective than MTZ in terms of lethal effect on *Blastocystis* sp. cells, which is confirmed by the actual MLA values established for these compounds: 125 μg/ml and 500 μg/ml, respectively ($p < 0.05$).

3. In contrast to MTZ, subinhibitory concentrations of BLA (≤ 2 μg/ml) do not stimulate the proliferation of *Blastocystis* sp. cells and, compared to MTZ, increase the virulent potential of parasites by the PAF index ($p < 0.05$) to a 1.6-fold lesser extent with a comparable effect on the growth of the PA index. Based on the results of the study, BLA was recognized as a promising compound for further research aimed at developing a more effective drug than MTX for the treatment of blastocystosis.

Sensitivity of *Blastocystis* sp. clinical strains to new benzimidazol derivatives

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Introduction. *Blastocystis* sp. (formerly *Blastocystis hominis*) is the most common protists of the intestinal tract of humans and many species of animals. *Blastocystis* sp. can cause various diseases of the digestive organs, which are currently combined into a separate nosology called "blastocystosis". To date, there are no international or national recommendations for the treatment of blastocystosis, including approved drugs for this purpose. Data on the clinical and parasitological effectiveness of various drugs used to treat blastocystosis are controversial. This substantiates the relevance of the search for new compounds with pronounced anti-*Blastocystis* activity for the development of drugs based on them for the etiotropic therapy of blastocystosis. The **goal** of this study was to determine the *in vitro* sensitivity of clinical strains of *Blastocystis* sp. to new derivatives of benzimidazole in comparison with metronidazole as well as to investigate the effect of the subinhibitory concentration of the most promising benzimidazole derivative on the level of parasite virulence factors (formation of amoeboid forms and production of proteases). **Materials and Methods.** Five cultures of *Blastocystis* sp. were isolated from faecal samples of patients with irritable bowel syndrome with predominant diarrhoea (IBS-D, Rome IV). Strains of *Blastocystis* sp. was cultured at 37 °C under anaerobic conditions in tubes with containing 5 ml of RPMI-1640 liquid nutrient medium with L-glutamine and enclosed antibiotics (ampicillin 12 mg/ml, streptomycin 4 mg/ml) and 10 % heat-inactivated serum of horse. The group of new benzimidazole derivatives (NBDs) included: tris-benzimidazole of aconitic acid (BAA), benzimidazole of histidine (BHI), benzimidazole of lipoic acid (BLA) and bis-benzimidazole of lysine succinamide (BBL). The anti-*Blastocystis* activity of NBDs and metronidazole (MTZ) was evaluated in the range of their concentrations from 1000 μg/ml to 1 μg/ml for four days with daily (after 24 h, 48 h, 72 h and 96 h) determination of indicators for each drug: 50 % of the minimum inhibitory concentration (MIC_{50}) and the minimum lethal concentration (MLC). Cell count of *Blastocystis* sp. was performed in a hemocytometer using the trypan blue dye exclusion test. Morphological changes in *Blastocystis* sp. cells induced by NBDs described according to the results of their phase-contrast microscopy. The effect of subinhibitory concentrations of NBD and MTZ on the formation of amoeboid forms (PAF) of *Blastocystis* sp. was established by counting the specific proportion (%) of these forms among 300 parasite cells in smears of suspensions permanently stained by the modified method according to Field. The level of protease activity (PA) in *Blastocystis* sp. cell lysates grown both in intact RPMI medium and in the presence of subinhibitory concentrations of NPB and MTZ was determined by the method of quantitative colorimetric analysis of azocasein cleavage. **Results and Discussion**. Based on the results of *in vitro* sensitivity studies of 5 clinical strains of *Blastocystis* sp. to the action of 4 NBDs (BAA, BHI, BLA and BBL) and metronidazole (MTZ), it was established that the anti-*Blastocystis* activity of BAA, BHI, BLA and MTZ reflects a direct positive regularity in the "concentration-response" and "contact time-response". That is, as the concentration of these benzimidazole derivatives increases, as well as the time of their action on *Blastocystis* sp. cultures, the manifestations of growth inhibition and death of parasite cells clearly increase. Instead, it was found that the effect of different concentrations of BBL on strains of *Blastocystis* sp., in the context of the "concentration-response" effect, is variable - with different directions of manifestations of this effect. In the stationary growth phase of *Blastocystis* sp. (72-hour parasite cultures) the MIC_{50} value for BLA was (7.2 ± 2.6) μg/ml and was 1.5 times, 1.6 times, 21.5 times and 1.7 times lower than the level of this indicator for BAA, BHI, BBL and MTZ, respectively ($p < 0.05$). In addition, BLA is 4 times superior to MTZ in the effectiveness of lethal action on *Blastocystis* sp. cells, which confirms the actual MLC values established for these compounds: 125 μg/ml and 500 μg/ml, respectively ($p < 0.05$). We discovered a deep destruction of cells of *Blastocystis* sp., which occurs when the parasite cultures are exposed to lethal concentrations of BLA: the central body disappears in the cells, intensive formation of granules and vacuoles is observed, thinning and loss of a clear contour of the outer shell, which is finally completely destroyed, and in place of the cells the detritus of their internal contents is revealed. Unlike MTZ, subinhibitory concentrations of BLA (≤ 2μg/ml) do not stimulate the reproduction of *Blastocystis* sp cells. Subinhibitory concentrations of BLA, compared to MTZ, increase the virulence potential of parasites to a lesser extent by 1.6 times according to the PAF indicator ($p < 0.05$) with a comparable effect of both ingredients on increasing the PA index.

Conclusion. The obtained results make it possible to recognize BLA as a promising compound for further research aimed at the development of a more effective means than MTZ for the treatment of blastocystosis.

Keywords: anti-*Blastocystis* activity, new benzimimidazol derivatives, metronidazole

DOI: 10.5281/zenodo.8046133

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