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VERSITA

Anti-*Pentatrichomonas hominis* activity of newly synthesized benzimidazole derivatives – *in vitro* studies

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Abstract

Pentatrichomonas hominis, a parasitic protozoan often detected in human diarrheic stools, is the cause of severe morbidity in newborns and children, particularly in tropical zones. The flagellate is resistant to many disinfectants and anti-protozoan drugs. Therefore in this study we have synthesized three novel 4,5,6,7-tetrabromobenzimidazole (TBBI) derivatives carrying a polyfluoroalkyl substituent in position 2 of the benzimidazole scaffold, namely 2-trifluoromethyl-TBBI (CF_3-TBBI), 2-nonafluorobutyl-TBBI (C_4F_9-TBBI), and 2-nonadecafluorononyl-TBBI (C_9F_{19}-TBBI), that next we tested for their *in vitro* activity against *P. hominis*. Widely applied anti-protozoal drug, metronidazole as a reference was used. All the investigated agents were added to 24 h *P. hominis* cultures; each of them was administered at three different concentrations. Number of the moving trichomonads was determined and compared with the control cultures. Different anti-trichomonal activity occurred depending on a kind of compound and its concentration. C_4F_9-TBBI was the most effective TBBI derivative tested: the agent, at the highest concentration 24.2 µg/ml, after 72 h reduced the number of viable trichomonads to 44.3%; C_9F_{19}-TBBI, at the concentration 24 µg/ml reduced the number of the flagellates to 58.5%. Paradoxically, metronidazole after the same time given at the highest concentration increased trophozoite counts by 464.6% in comparison with the control cultures (100%).

Keywords

Pentatrichomonas hominis, benzimidazole derivatives, metronidazole, anti-trichomonal activity

Introduction

Pentatrichomonas hominis (Davaine, 1860) Wenrich, 1931 (Trichomonadidae) is a worldwide occurring extracellular protozoan residing in the distal part of the small intestine and large intestine of man and other primates as well as dogs and cats. Although a pathogenic impact of this trichomonad is still discussed, it is known, that this flagellate is the causative organism of trichomonosis of the digestive tract and is often identified in human diarrheic stools (Yang *et al*. 1990, Chomicz *et al*. 2004, Guillaume 2007). In Poland, the prevalence of *P. hominis* is estimated to be above 2% but in subtropical and tropical zones it is much higher, up to 40% (Saksirisampant *et al*. 2003). Increased prevalence of the protozoan is usually directly associated with poor social-economic conditions. Because of enhanced tourist traffic, *P. hominis* could be more often transmitted from tropical regions into Europe (Petersen 1988, Chomicz *et al*. 2004, Górska *et al*. 2006).

Infections with *P. hominis* are more common in children than in adults mainly due to fecal-oral transmission route of the trichomonad. Severe diarrhea cases associated with *P. hominis* have been reported in children up to five years of age (Yang *et al*. 1990, Chunge *et al*. 1992). Prolonged infection of this trichomonad in newborns can cause wasting diarrhea – one of the important medical problems in children (Mancilla-Ramirez and Gonzalez-Yunes 1989). A case of *P. hominis* infection was reported in a lymphoma patient with severe diarrhea (Shaio *et al*. 1981). It could also cause a serious

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condition in people and other hosts with impaired immune response.

It was reported that *P. hominis* infection might display a pathogenic course in rats after splenectomy or after stress which causes immunological problems of the host (Al-Dabagh and Shafiq 1970). The trichomonad was also found in areas other than the gastrointestinal tract, i.e. it has been sometimes found in liver abscesses (Jacobsen *et al*. 1987, Honigberg 1990) as well as in vaginal specimens (Crucitti *et al*. 2004). A fatal case of systemic lupus erythematosus in a woman harboring *P. hominis* in both stool and exudative pleural effusion was reported (Jongwutiwes *et al*. 2000); identification of the flagellate has been confirmed in this patient by DNA sequencing. Many cases of mixed infections with *P. hominis* and other protozoa, such as *Entamoeba histolytica*, *Giardia intestinalis*, or with bacteria such as *Campylobacter*, *Shigella* were also described (Reinthalter *et al*. 1988, Chunge *et al*. 1992). The zoonotic potential of the flagellate remains to be determined.

Although in many developing countries *P. hominis* is the most commonly found flagellate next to *Giardia intestinalis*, this protozoan is still poorly described; no prevention nor optimal treatment has been defined (Crucitti *et al*. 2004, Górska *et al*. 2006).

The antimicrobial activities of imidazoles have been known for long. Benzimidazole derivatives can be active against bacteria, parasites as well as fungi and viruses (Alunni Bistocchi *et al*. 1986, Sheehan *et al*. 1999, Upcroft *et al*. 1999, Kazimierczuk *et al*. 2002, Kopańska *et al*. 2004, Chomicz *et al*. 2005). Metronidazole, one of the most popular nitroimidazole drugs, is widely used against many protozoan parasites (Cedillo-Rivera and Muñoz 1992, Wassmann and Bruchhaus 2000, Cedillo-Rivera *et al*. 2002, Chomicz *et al*. 2005). It has been the main drug of choice for treatment of trichomonosis caused by *T. vaginalis s*ince 1959. Because of the increased use of the drug, more and more strains of parasitic protozoa have become resistant to metronidazole (Land and Johnson 1999, Wassmann and Bruchhaus 2000, Blaha *et al*. 2006). In addition, many patients cannot tolerate these high doses of metronidazole, and some – even low concentrations of the drug (Borchardt *et al*. 1996, Narcisi and Secor 1996, Schwebke and Burgess 2004). The increase of adverse events in treated patients is reported, including: neuropathy, gastrointestinal reactions, mutagenic and teratogenic effects (Kasten 1999).

The anti-protozoal activities of benzimidazole derivatives, especially against *P. hominis*, have not been extensively investigated. Because treatment failures with nitroimidazole derivative – metronidazole more frequently occur as well as the number of resistant strains of protozoa is increasing, there is a need for search of new anti-protozoal agents. In this study new tetrabromine-substituted fluoroalkylbenzimidazole derivatives were prepared and tested as prospective chemicals against trichomonads. Particularly, the purpose of these studies was to examine *in vitro* the effects of different concentrations of the newly synthesized benzimidazole derivatives on the viability of trophozoites of *P. hominis*.

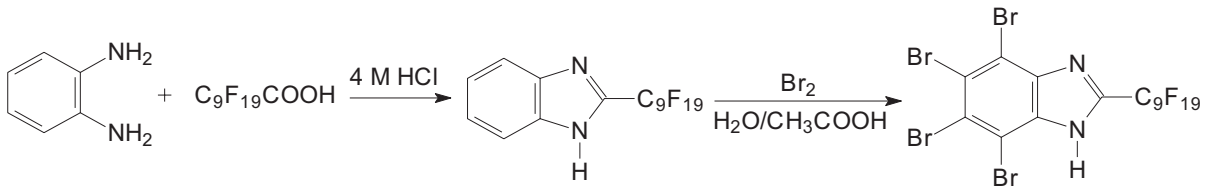
Materials and methods

The preparation of the benzimidazole derivatives

The corresponding 4,5,6,7-tetrabromo-2-polyfluoroalkylbenzimidazoles: 2-trifluoromethyl-4,5,6,7-tetrabromobenzimidazole (CF_3-TBBI) and 2-nonafluoro-4,5,6,7-tetrabromobenzimidazole (C_4F_9-TBBI) were prepared following the reported method (Büchel 1970, Andrzejewska *et al*. 2003). The synthesis of 4,5,6,7-tetrabromo-2-nonadecafluorononylbenzimidazole (C_9F_{19}-TBBI) was performed by the bromination of 2-nonadecafluorononylbenzimidazole according to following procedures.

Synthesis of 2-nonadecafluorononylbenzimidazole. The mixture containing o-phenylene diamine (2.0 g, 18.5 mmol) in 4 M HCl (45 ml) and nonadecafluorodecanoic acid (22 mmol) was heated under reflux for 5 days. The resulting solution was evaporated to dryness, diluted with water and ethanol, treated with charcoal and brought to pH 3–4. The precipitate formed was filtered off and crystallized from ethanol-water (1:1, v/v). Yield: 27%; m.p. 196–198°C.

Synthesis of 4,5,6,7-tetrabromo-2-nonadecafluorononylbenzimidazole, C_9F_{19}-TBBI.



To the stirred and refluxed suspension of the 2-nonadecafluorononylbenzimidazole (1.5 g, 2.56 mmol) in a mixture of water (60 ml) and acetic acid (60 ml), bromine (5 ml, 97.5 mmol) was added portionwise within 7 h. The reflux was continued for 3 days. The reaction mixture was cooled and the orange precipitate was filtered off, washed with water and crystallized from ethanol-water (1:1, v/v). Yield: 69%; m.p. 187–190°C.

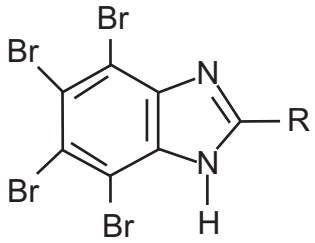
^{1}H-NMR and UV-spectra as well as elemental analyses are available from the authors upon request.

All chemicals and solvents were purchased from Sigma-Aldrich. Melting points were determined in open capillary tubes on a Gallenkamp-5 melting point apparatus and were uncorrected. UV spectra were recorded in a Kontron Uvikon 940 spectrophotometer. ^{1}H-NMR spectra (in ppm) were measured

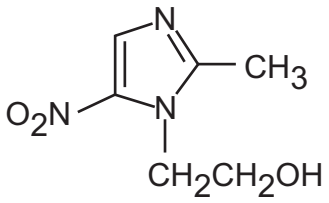
with a Varian Gemini 200 MHz (or a Varian UNITY plus 500 MHz) spectrometer at 298 K in DMSO-d_6 using TMS as internal standard. Mass spectra (70 eV) were obtained with an AMD-604 (Intectra) spectrometer. Flash chromatography was performed with Merck silica gel 60 (200–400 mesh). Analytical TLC was carried out on precoated silica gel F_{254} (Merck) plates (0.25 mm thickness). Analyses of the new compounds, indicated by the symbols of the elements, were within ± 0.4% of the theoretical values.

Metronidazole was purchased from Sigma-Aldrich.

Chemical structures of all studied new polyfluorobenzimidazole derivatives and metronidazole.



R_1-CF_3 (4,5,6,7-tetrabromo-2-trifluoromethylbenzimidazole)
R_2-C_4F_9 (4,5,6,7-tetrabromo-2-nonafluorobuthylbenzimidazole)
R_3-C_9F_{19} (4,5,6,7-tetrabromo-2-nonadekafluorononylbenzimidazole)



Metronidazole: 1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole.

Trichomonads studied

Trophozoites of *Pentatrichomonas hominis* isolated from diarrheic stool of an adult patient were grown at 37°C in 15 ml tubes containing the liquid Pahm medium (Myjak 1974) and subcultured twice a week. The medium was used in hospital immediately when the diarrheic stool sample was taken from patient, thus further investigations on the strain of *P. hominis* have been conducted with use of the same medium. Pahm medium contains mixture of phosphate buffer solution ($NaHPO_4 \times H_2O$ 4.77 g, KH_2PO_4 1.81 g, NaCl 32 g and redistilled water 4 l, pH 6.8) and MSF base (casein hydrolysate 8 g, glucose 4 g, NaCl 1 g, KH_2PO_4 0.28 g, cysteine hydrochloride 0.32 g, thiomalic acid 0.6 g, piridoxal hydrochloride 0.002 g, redistilled water 400 ml, the mixture was adjusted to pH 6.8 with 10% NaOH sol.). The mixture of the both agents in ratio 3400 ml (buffer) to 400 ml (MSF base) was autoclaved for 30 min. To the chilled mixture horse serum (50 ml) and sterile rice starch (240 g) were added. The Pahm medium was portioned and kept in 4°C for further use.

Assessment of the developmental dynamics of the trichomonad population showed that one-day cultures were in the growth phase, thus cultures at the 24 h following regular subculturing containing 2–4 × 10^4 cells ml^{-1} were used to test susceptibility of trichomonads to all novel compounds and metronidazole. Halogenobenzimidazoles were dissolved in 25% ethanol and 1 M NaOH in proportion of 1:5, whereas metronidazole was dissolved in dimethylsulfoxide (DMSO). After the transfer of 1 ml cultures of *P. hominis* to individual 1.5 ml Eppendorf tubes, 10 µl of each agent solution were added to parts of those tubes. Three final concentrations of each tested benzimidazoles and metronidazole were used (respective concentrations in µg/ml and in µM are given in Table I). Dissolvent and negative control assays were also performed. All experiments were conducted at 37°C. Before the compounds and/or dissolvent were added to the tubes with one-day trichomonad cultures (0 h of exposure), shape and motility of the protozoans were assessed microscopically; the Bürker chamber was used to determine number of the living protozoans. Further observations and counts were made after 24, 48 and 72 h for cultures exposed to the tested substances at 37°C and for the controls. Mean values of 4–8 counts calculated for 1 ml of culture medium were counted and compared with those of respective control cultures. Specific reaction of *P. hominis* to the compounds tested enabled to assess the percentage of viable trophozoites in each interval rather than minimum inhibitory concentrations (MIC) considered to be less representative. Percentages of moving protozoans were determined in comparison with the results of the control assays (100%) and analyzed statistically (ANOVA, Student-Newman-Keuls method, $\alpha \leq 0.05$).

Results

Comparative assessment of the results of the studies showed that all compounds tested caused changes in development dynamics of the *P. hominis* strain investigated by us. Microscopic examination of the trichomonads, both of the control cultures and those exposed to chemicals revealed some differences between treated and untreated protozoans. Some changes in movement and appearance of the trophozoites were observed already after 24 h in all assays with compounds: some of the living trichomonads moved more slowly, and rounded forms were visible more frequently than in the control cultures.

Pentatrichomonas hominis trophozoites showed great variation in a susceptibility to the chemicals tested. In the experiments with the fluoroalkylbenzimidazole derivatives, CF_3-TBBI indicated a slight activity against the protozoans. The C_4F_9-TBBI and C_9F_{19}-TBBI were more effective: clear antitrichomonal activity was manifested stronger at higher concentrations of the agents, 24 µg/ml. After 24 h exposure, C_4F_9-TBBI clearly reduced number of moving *P. hominis*

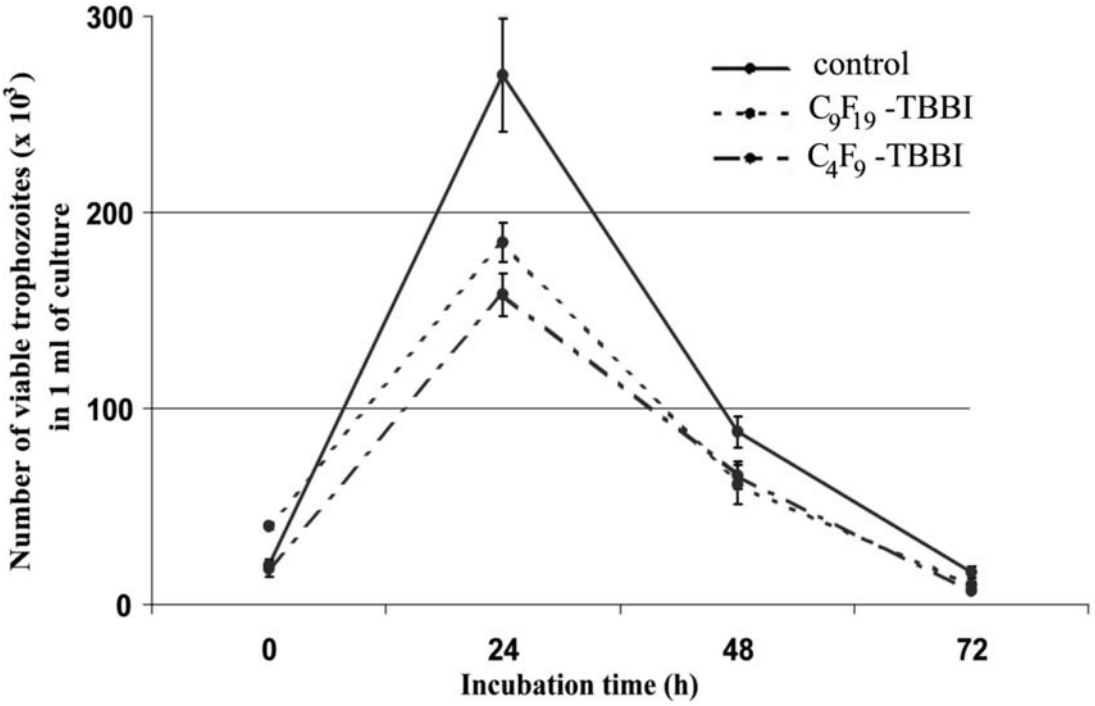


Fig. 1. The anti-trichomonal effect of C_4F_9-TBBI and C_9F_{19}-TBBI on the grown of *P. hominis* trophozoites in comparison to the control culture

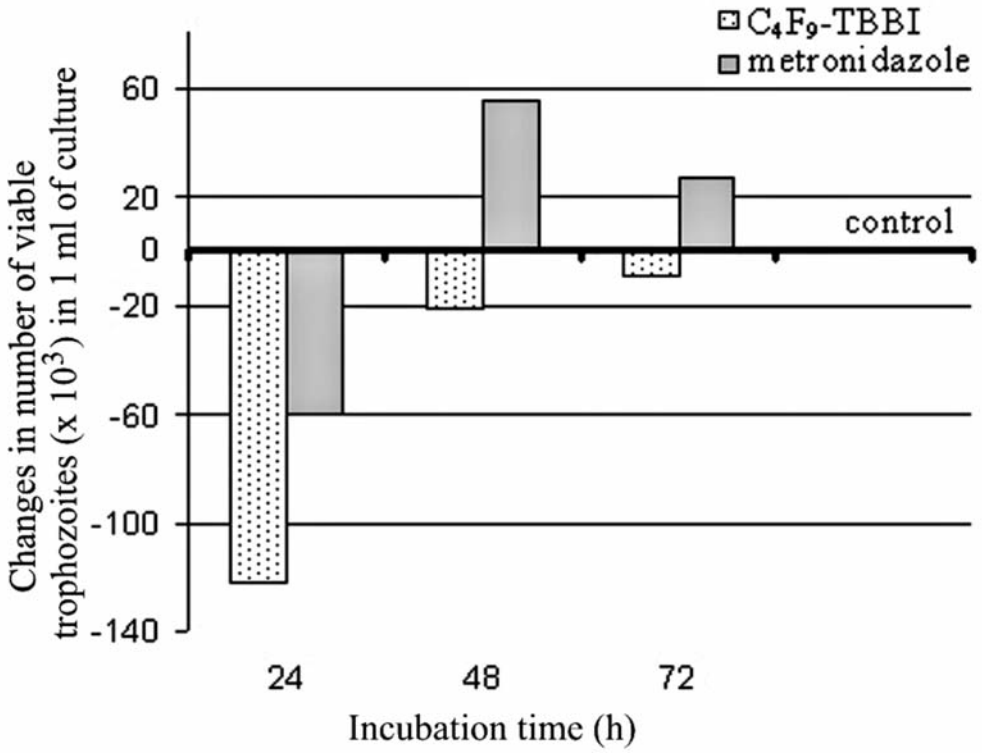


Fig. 2. Changes in number of *P. hominis* trophozoites after application of metronidazole (48 μM) and C_4F_9-TBBI (38 μM) in comparison to the respective control cultures – level 0

Table I. Percentages of viable *P. hominis* after 24 h, 48 h and 72 h exposure to the different concentrations of the compounds tested, in comparison with the control assays (100%)

Time	Concentration of compounds µg/ml (µM)											
	CF_3-TBBI			C_4F_9-TBBI			C_9F_{19}-TBBI			metronidazole		
	4.0 (8)	12.1 (25)	24.0 (48)	4.0 (6)	12.0 (18)	24.2 (38)	3.8 (4)	12.1 (14)	24.0 (25)	4.2 (25)	8.2 (48)	12.0 (70)
24 h	103.6	108	113.4	75.6	71.5	55.3	87.2	99.4	95.4	97.2	78.7	24.6
48 h	87.8	75	67	88	75.8	75	119.5	95.5	59.2	118.7	163.6	86.8
72 h	68.5	71.4	95.7	95.4	69.5	44.3	114.2	102.8	58.5	172.8	266.7	464.6

Data significantly different ($\alpha \leq 0.05$) from that in the respective control cultures are underlined.

trophozoites. At higher concentration of the agent, 24.2 µg/ml, the percentage of viable trichomonads was decreased to 55.3. The compound C_9F_{19}-TBBI, containing the longer polyfluoroalkyl substituent, was not as effective after 24 h of incubation as C_4F_9-TBBI, and its activity against the trichomonads was higher at lower concentrations of the agent, 3.8 µg/ml: the percentage of viable trichomonads was decreased to 87.2 in comparison with the respective control culture (100%).

Finally, after 72 h exposure, statistically significant lower number of trophozoites was observed at higher concentrations of the newly synthesized benzimidazoles.

C_4F_9-TBBI was *in vitro* the most effective against *P. hominis* among all newly synthesized fluoroalkylbenzimidazole derivatives examined in this study. The compound at the highest concentration, 24.2 µg/ml, reduced the number of viable protozoans to 44.3% in comparison with the number of the flagellates that has been detected in respective control culture (100%). C_9F_{19}-TBBI also indicated clear anti-*P. hominis* activity. After 72 h exposure to higher concentration of the agent, 25 µM/l, the percentage of viable trichomonads was decreased to 58.5, as compared to the control culture. The anti-trichomonal effects of C_4F_9-TBBI and C_9F_{19}-TBBI have been illustrated in Figure 1.

In experiments with metronidazole, after 24 h exposure to low concentrations of the drug, a slight activity against *P. hominis* was apparent. The strongest reaction occurred when this agent in the highest concentration, 12 µg/ml, was applied.

In spite of this anti-trichomonal tendency observed after 24 h exposure, finally, after 72 h incubation, in all assays with addition of metronidazole more protozoans was found than in the respective control cultures. This surprising effect was particularly expressive at the highest concentration of metronidazole, 12 µg/ml: the number of the trichomonads reached 464.6% as compared to the control cultures (100%). Changes in number of *P. hominis* trophozoites after application of metronidazole and C_4F_9-TBBI, in comparison to the control cultures, designed as level 0 have been presented in Figure 2.

The comparison of percentages of the moving *P. hominis* trophozoites that have been detected in several assays after 24, 48 and 72 h exposure to the different concentrations of the three newly synthesized fluoroalkylbenzimidazole derivatives and metronidazole, in comparison with those of the control assays have been presented in Table I.

Discussion

It is known that 4,5,6,7-tetrabromobenzimidazole (TBBI) is a highly selective inhibitor of casein kinase 2 (CK2) and of the NTPase/helicase from hepatitis C virus and West Nile virus (Kopańska *et al*. 2004). Fluoroalkylbenzimidazoles are potent decouplers of oxidative phosphorylation in mitochondria, and they inhibit photosynthesis. Antibacterial and antifungal activity was also observed. Benzimidazoles containing carbamate-residue in position 2 and an H-atom in position 1, bind to tubulin and thereby inhibit its polymerization. A strong activity of halogenobenzimidazoles against *Trichomonas vaginalis*, *Giardia intestinalis* and *Entamoeba histolytica* (all of them have a tubulin cytoskeleton) was revealed in the earlier studies (Andrzejewska *et al*. 2002, Kazimierczuk *et al*. 2002).

The results obtained from these *in vitro* studies on the influence of the newly synthesized benzimidazole compounds showed that brominesubstituted 2-fluoroalkylbenzimidazoles, particularly C_4F_9-TBBI and C_9F_{19}-TBBI are promising chemicals against *P. hominis*. The reduction of viability of the trichomonad population was expressed as a distinct decrease in average number of the moving trophozoites as well as significant changes in appearance and movement of the flagellates. Simultaneously, in this study, after 72 h exposure of *P. hominis* to different concentrations of metronidazole, statistically significant higher numbers of the protozoans have been found in all assays to which the drug was added, as compared to the control cultures. Although it was not know if metronidazole has been absorbed by the *P. hominis* protozoan cells, it was likely, that, with passage of the time, changes in the drug concentration occurred; simultaneously, a resistance to metronidazole of the trichomonad population studied by us cannot be excluded.

The first case on unsuccessful treatment with metronidazole in human urogenital trichomonosis was described by Robinson (1962). Since that time, many cases of resistance of parasitic protozoans to this drug have been recorded (Meingassner and Thurner 1979, Munoz *et al*. 1998). The intracellular mechanism of the anti-protozoal activity of metronidazole is known: the drug enters the cell by passive diffusion and only becomes activated within the cell. In bacteria and in some protozoa (*Giardia*, *Entamoeba*) activation of metronidazole takes place in cytoplasm and in *Trichomonas* within specialized organelles, hydrogenosomes. Inside the protozoan cell, the reduction of nitro group of metronidazole occurs by reduced ferredoxin or flavodoxin to form a reactive nitro radical. The cytotoxic influences on different protozoa species inflicted by metronidazole are believed to be involved inhibition of nucleic acid synthesis or damage the DNA of parasitic cell (Ings *et al*. 1974).

Resistance to metronidazole has been demonstrated *in vitro* for *G. intestinalis*, *E. histolytica* and *T. vaginalis* (Land and Johnson 1999, Wassmann *et al*. 1999, Wassmann and Bruchhaus 2000). It has been suggested that the drug resistance of *E. histolytica* was the result of modification of proteins involved in drug activation, i.e. associated with increased expression of iron-containing superoxide dismutase and decreased expression of ferredoxin 1 and flavin reductase. In trichomonads, a resistance to metronidazole has been observed in aerobic and anaerobic conditions, both in the natural and experimental circumstances (Kulda 1999, Meri *et al*. 2000, Rasoloson *et al*. 2001). The first type occurs when impaired oxygen scavenging leads to re-oxidation of nitro radicals or when electrons are competitively removed by oxygen. The so-called anaerobic resistance developed after long periods of incubation with increasing concentrations of metronidazole; it was explained as an elimination of drug-activating pathway inside the protozoan cell by the gradual decrease and/or loss of the ferredoxin oxidoreductase. The increase of the moving trichomonads induced by metronidazole was sometimes observed in our earlier researches (Chomicz *et al*. 2004, Górska *et al*. 2006). The fact that metronidazole has been applied for more than 45 years by now, can explain metronidazole-resistant strains of bacteria and protozoans.

This is the first report presenting the results of our *in vitro* studies with use the three novel benzimidazole derivatives that would be promising as prospective chemicals against the trichomonad. It should be taken into consideration that *P. hominis* may be more frequent parasite in near future due to increasing number of persons with immunological dysfunctions or under chronic immunosuppression; it was pointed out that this trichomonad might be a potential intestinal pathogen particularly for immunocompromised hosts such as newborns and malnourished infants (Mancilla-Ramirez and Gonzalez-Yunes 1989). Thus, also for this reason, further studies on variously substituted halogenobenzimidazoles will be very helpful to determine the proper concentrations of the compounds for their higher efficacy against *P. hominis* as well as to assess the significance of other factors for resistance/susceptibility of the trichomonad to the chemicals.

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