Effects of three benzimidazoles on growth, general morphology and ultrastructure of *Tritrichomonas foetus*

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**Abstract**
*Tritrichomonas foetus* is a venereal pathogen of cattle, which causes infertility, early embryonic death or abortion. In order to evaluate the potential trichomonicidal activity of benzimidazoles, the effects of thiabendazole, mebendazole and albendazole were analyzed on the multiplication, general morphology and ultrastructure of *T. foetus*. It was found that mebendazole presented the highest IC₅₀% (2.3 μM), when compared with albendazole (IC₅₀% = 9.4 μM) and thiabendazole (IC₅₀% = 142.6 μM), and that such effects were irreversible. Concerning microscopic analysis, thiabendazole- and mebendazole-treated cells presented increased volume, internalization of the flagella, disruption or multiplication of the nucleus, multiple organelles and cytoplasmic vacuolization. Albendazole-treated cells exhibited slight alterations, because the parasite became slightly rounded, its flagella were not internalized but the cytoplasm was vacuolated. Mebendazole was indeed highly effective as an *in vitro* trichomonicidal agent, and this might open up new possibilities for the use of mebendazole in the therapy of bovine trichomoniasis.

**Introduction**
*Tritrichomonas foetus* is a flagellated parasitic protozoan that infects the bovine urogenital tract, where it causes a sexually transmitted disease known as bovine trichomoniasis. *Tritrichomonas foetus* has a worldwide distribution and causes significant economic losses attributed to infertility, early embryonic death or abortion in cattle (Fitzgerald, 1986; Yule et al., 1989; Speer & White, 1991; BonDurant & Honigberg, 1994). The parasite is transmitted from the prepuce of bulls to the vagina and uterus of cows, during coitus and its principal clinical manifestation is abortion during the first half of gestation (BonDurant, 1997). Recently, the parasite has also been described as an inhabitant of the porcine GI and nasal mucosa, and also found colonizing the colon of domestic cats (Yaeger & Gookin, 2005).

Unfortunately, there is no effective therapy against bovine trichomoniasis. Ipronidazole (Skirrow et al., 1985; Williams et al., 1987; Skirrow & Bondurant, 1988) and dimetridazole (McLoughlin, 1965, 1968, 1970; Kimsey et al., 1980; Campero et al., 1987) have been used but they are no longer available for use in cattle.

The search for new drugs to be used in the treatment of diseases caused by parasitic protozoa has led to a reevaluation of the benzimidazoles as antiprotozoan agents (Cedillo-Rivera & Munoz, 1992; Chavez et al., 1992; Oxberry et al., 1994; Liu & Weller, 1996; Skinner-Adams et al., 1997; Armson et al., 1999; Valdez et al., 2002; Bernal-Redondo et al., 2004).

Benzimidazoles have been widely used since the 1960s as antihelmintic agents in both veterinary and human medicine (Katiyar et al., 1994). It has been suggested that inside some parasites, the benzimidazoles greatly affect the colchicine binding-site of β-tubulin monomers inhibiting, therefore, microtubule assembly and disassembly (Lacey, 1988; Meloni et al., 1990; Magne et al., 1991). In addition, the benzimidazoles also play a role as lipid-soluble proton ionophores, inducing direct and indirect cellular biochemical changes including inhibition of glucose uptake,
glycogen depletion, inhibition of the fumarate reductase system and uncoupling of electron transport-associated phosphorylation (McCracken & Stillwell, 1991).

The aim of the present work is to further evaluate the effects of each one of benzimidazoles (thiabendazole, mebendazole and albendazole) on growth, general morphology and ultrastructure of T. foetus.

Materials and methods

Parasites

The K strain of T. foetus was used throughout. The parasites were cultivated in trypticase–yeast extract–maltose (TYM) Diamond’s medium (Diamond, 1957) supplemented with 10% heat inactivated fetal bovine serum (FBS) at 37°C, for 24 h. Just after, they were collected by centrifugation, washed twice with a 0.01 M phosphate-buffered 0.15 M NaCl solution (PBS) and assayed.

Drug susceptibility assays

Trophozoites (5×10⁴ parasites mL⁻¹) were inoculated in fresh TYM medium in the presence or not of 0.5, 1, 5 or 10 µg mL⁻¹ of albendazole or mebendazole or 1, 10, 50 and 100 µg mL⁻¹ of thiabendazole. Benzimidazoles (Sigma Chemical, St Louis, MO) were stored as 10 mg mL⁻¹ stock solutions made in 0.1% dimethyl sulfoxide (DMSO; Merck, Darmstadt, Germany). Following growth at 37°C, untreated and DMSO- or benzimidazoles-treated parasites were collected at 4, 6, 16 and 24 h, and counted in a Neubauer chamber. The benzimidazole concentration that inhibits parasite growth at 50% (IC₅₀) was evaluated for each treatment.

Reversibility test

Assays were done in order to evaluate if the effects exerted by the benzimidazoles in T. foetus could be reversed. Parasites were incubated with each one of the benzimidazoles as described above, for 6 or 24 h at 37°C. Some of the untreated and benzimidazole-treated parasites were fixed, and counted in a hemocytometer at the end of each incubation time. The others were inoculated in a drug-free fresh culture medium for 24 h. After that, parasite density and viability were recorded by counting erythrosine B-stained parasites.

Statistical analysis

The resulting values of drug susceptibility assays were expressed as mean ± SEM and the results were compared by repeated measures ANOVA, followed by Dunnett’s multiple comparision test when apropriate. The resulting data of reversibility tests were presented as percentage of controls ± SEM and were compared by the unpaired Student’s t-test. P-values < 0.05 were considered significant.

Video-microscopy

Untreated and benzimidazoles-treated parasites were mounted between a glass slide and coverslip, and observed using differential interference contrast (DIC) microscopy. Video-microscopy images were obtained in a Zeiss Axioshot microscope (Zeiss, Germany) equipped with a digital camera SIS CC-12 and analyzed with the ANALYSIS 3.1 software (SIS-Soft Imaging System, Munster, Germany). The resulting images were optimized with the use of the COREL PHOTO PAINT 11 software (Corel Coporation, 2006).

Transmission electron microscopy (TEM)

Untreated and benzimidazoles-treated parasites were sequentially fixed for 2 h at room temperature in a solution containing 2.5% glutaraldehyde, 4% paraformaldehyde, 4% sucrose and 5 mM CaCl₂ in PHEM buffer (PIPES–HEPES–EGTA–MgCl₂, pH 7.2) (Schliwa & van Blerkom, 1981), rinsed in the same buffer, postfixed for 30 min in 1% OsO₄ made in PBS, at 4°C, and rinsed once with PBS. The parasites were then incubated for 30 min in 1% tannic acid made in PBS, dehydrated in acetone and embedded in Epon (Ted Pella Inc., Redding, CA). Finally, the resulting ultrathin sections obtained were collected in copper grids and contrasted in 3% aqueous uranyl acetate and lead citrate and then observed in a Zeiss EM906 TEM (LEO Electron Microscopy GmbH, Oberkochen, Germany).

Results

Growth inhibition

All the benzimidazoles tested here inhibited the in vitro growth of T. foetus to different extents. The inhibitory effects induced by benzimidazoles, whose kinetics are shown in Fig. 1a–c, reveal that treatment with thiabendazole inhibited significantly parasite growth at concentrations ≥ 50 µg mL⁻¹ from 4 h of incubation. A significant growth inhibition of T. foetus was observed when it was incubated with 0.5 µg mL⁻¹ mebendazole for 4 h at least. Finally, albendazole was able to induce an expressive growth inhibition of T. foetus from 5 µg mL⁻¹ after 4 h of incubation.

The IC₅₀ obtained for each one of the benzimidazoles is shown in Table 1. It was found that mebendazole was able to inhibit growth at 50% after only 4 h of incubation with 14.9 µM and presented a minimum IC₅₀ of 2.3 µM in 24 h. Both albendazole and thiabendazole presented a good trichomoncidal activity with IC₅₀ of 9.4 µM (24 h) and 142.6 µM (24 h), respectively.
Reversibility tests

Even when benzimidazoles-treated parasites (6 or 24 h) were incubated in benzimidazoles-free fresh culture medium for 24 h, most of parasites did not restore their original growth. Just a low percentage of viable parasites was detected (Fig 2). As can be seen in Fig. 2, the number of parasites submitted to the treatments followed by incubation in a drug-free medium (T1DFM) was reduced in comparison with each of the controls (black bars) and with those counted immediately after treatment (T). The viability test demonstrated that the remaining cells were still viable, although they were not able to perform mitosis (data not shown).

Video-microscopy

The general morphology of an untreated trichomonad, which is shown in Fig. 3a, reveals a pear-shaped body containing structures as the anterior flagella emerging from the anterior end of the parasite (circle) and the axostyle (arrow). By contrast, thiabendazole- or mebendazole-treated T. foetus presents intense alterations in its general morphology. Thiabendazole-treated T. foetus became rounded and vacuolated (Fig. 3b). After 24 h of thiabendazole treatment, most such giant parasites also exhibited two nuclei (Fig. 3b). The flagella were internalized after 24 h of treatment of T. foetus with thiabendazole. However, it was observed that some flagella continued to beat inside the parasite for a short period. In some cases, the thiabendazole-treated cells presented an increased number of flagella, which were not internalized (Fig 3b – arrows). About 70% of the parasites were observed as giant forms after 24 h incubation with 100 μg mL⁻¹ thiabendazole. Mebendazole-treated parasites (Fig. 3c) also became rounded and did not exhibit motility. Many of such treated parasites exhibited internalized flagella, while their cytoplasms appeared wrinkled. Large vacuoles were also seen in most of the mebendazole-treated parasites. Furthermore, after 24 h of treatment with 100 μg mL⁻¹ mebendazole, more than 90% of the parasites presented drastically altered morphologies.

By contrast, the treatment of T. foetus with different amounts of albendazole did not induce further morphological alteration in the parasite (Fig. 3d). Some parasites became slightly rounded after such treatment, and several vesicles could be observed in their cytoplasms.
all the flagella remained externalized (arrows), including the recurrent one.

**TEM**

The general fine structure of *T. foetus* can be seen in Fig. 4a. In such a longitudinal section of *T. foetus*, the flagella (black arrow), nucleus (n), hydrogenosomes (h), and glycogen particles (white circle) can be observed. The spherical hydrogenosomes (Fig. 4b) are surrounded by two closely adjacent membranes, and a vesicle-like structure was found between the two membranes.

Parasites incubated with different concentrations of thiabendazole presented severe morphological alterations, as already seen by video-microscopy. Few parasites showed their usual tear shape: most were rounded. In addition, many flagella were observed inside vesicles, which are in turn surrounded by membrane (Fig. 4c – arrows). The presence of hydrogenosomes with altered morphologies was also observed in such parasites. These organelles seem to be

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**Fig. 2.** Reversibility assays. Number of parasites counted immediately after treatment (six or 24 – T) or after treatment and further incubation in a drug-free medium for 24 h (six or 24 – T + DFM). (a) Thiabendazole – 1–100 μg mL⁻¹; (b) Mebendazole – 0.5–10 μg mL⁻¹; (c) Albendazole – 0.5–10 μg mL⁻¹. Values are expressed as percentages of control (n = 5) and bars represent SE. The unpaired Student’s t-test was used to compare the groups (NS, not significant; *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001).
oversized (2.0 μm diameter) and presenting a nonhomogeneous matrix (Fig. 4c – black circle). Much of thiabendazole-treated parasites presented multiple axostyles (white arrows), two nuclei (n) and some of their hydrogenosomes were found randomly dispersed over the cytoplasm (Fig. 4d), without exhibiting the usual association with both axostyle and costa.

The round-shaped mebendazole-treated parasites presented an intense cytoplasmic vacuolization (Fig. 4e) that could be seen in detail by TEM. These vacuoles varied in morphology and size and presented internal membranaceous structures (Fig. 4e – arrows). Profiles of endoplasmic reticulum were frequently seen in association with abnormal hydrogenosomes and vacuoles (Fig. 4f). Like those observed in thiabendazole-treated T. foetus, the axostyle remained polymerized even after a 24 h treatment of the parasites with mebendazole.

Using video-microscopy, no further morphological alteration was found in albendazole-treated parasites. However, using TEM it was observed that such parasites were ultrastructurally affected by the drug treatment, showing an intense cytoplasmic vacuolization (Fig. 4g). These vacuoles also varied in shape and size, presenting internal membranaceous profiles (Fig. 4h – arrows). Different from the other two drug-treated parasites, albendazole-treated parasites did not exhibit internalization of their flagella (Fig. 4g – black arrow).

**Discussion**

Members of the benzimidazole family have been widely used in both veterinary and medical clinics due to their mild side effects. They were first described as powerful antihelmintic agents (Bardon et al., 1995; Nontasut et al., 1997; Nakaya et al., 1998; Legesse et al., 2002, 2004; Mentz et al., 2004) and subsequently it was demonstrated that some benzimidazoles also presented a high efficacy against many parasitic protozoa (Edlind et al., 1990; Cedillo-Rivera & Munoz, 1992; Chavez et al., 1992; Oxberry et al., 1994; Liu & Weller, 1996; Skinner-Adams et al., 1997; Valdez et al., 2002), including some trichomonads (Juliano et al., 1985; Al-Waili, 1987; Sears & O’Hare, 1988). Of particular interest is the fact that still there is no effective chemotherapy against T. foetus.

The present study describes the trichomonicidal activity of three members of the benzimidazole family. The effects of thiabendazole, mebendazole and albendazole on growth, general morphology and ultrastructure of T. foetus were investigated.
All the benzimidazoles have inhibited significantly the in vitro growth of *T. foetus*. According to the parameters of Sawangjaroen *et al.* (2005), mebendazole presented the highest inhibitory activity with an IC$_{50\%}$ of 2.3 μM after incubation for 24 h. Mebendazole induced a growth inhibition rate in the order of 97% at 10 μg mL$^{-1}$ for 24 h. It is important to notice that all the drug treatments resulted in a loss of the ability of the parasites to restore their growth. Otherwise, the results of the viability test demonstrated that the remaining parasites were still viable, although they were not able to perform mitosis. Juliano *et al.* (1985) have previously demonstrated that some benzimidazoles irreversibly inhibited the growth of *Trichomonas vaginalis*. These authors did argue that such irreversible effects might be due to further damage in the nuclear structures of *T. vaginalis* (Juliano *et al.*, 1985). The same effect was observed in slime mouds (Wright *et al.*, 1976), fungi (Heat, 1982) and some...
other organisms submitted to treatment with benzimidazoles.

Using video-microscopy and TEM it was possible to analyze the effects of benzimidazoles on the general morphology and ultrastructure of *T. foetus*.

By video-microscopy analysis it was observed that among thiabendazole-treated cells most of them drastically changed their morphologies, presenting increased volume and some multiplicated organelles. Several authors have observed such abnormal forms when *T. foetus* was under drug pressure (Mariante *et al.*, 2003, 2006; Pereira-Neves *et al.*, 2003; Madeiro-da-Costa & Benchimol, 2004). No motility was observed in such treated parasites because most of them presented internalized flagellae into endocytic vacuoles. This process of flagella internalization might represent a parasite protection against the drugs. Granger *et al.* (2000) described previously that the flagella of *T. foetus* were internalized in an intact and functional form when parasites were submitted to stress conditions, such as variations in the environmental temperature. Using TEM, profound alterations in the ultrastructure of such drug-treated *T. foetus* could be observed. Many of the organelles were present in multiple copies and sometimes they seem to be damaged. Altogether, these results strongly suggest that treatment with thiabendazole did not block organelle division in *T. foetus*, but arrest parasite cytokinesis. Madeiro-da-Costa & Benchimol (2004) have found similar morphological alterations in *T. foetus* treated with the microtubular-affecting drug noco-dazole, which is in turn a member of the benzimidazole family. Concerning hydrogenosomes, treatment with thiabendazole induced a random distribution of these organelles and the appearance of an irregular hydrogenosomal matrix, as well as inducing a significant increase in volume. When the diameter of normal hydrogenosomes (0.3–0.5 μm) was compared with those of drug-treated parasites (2.0 μm), it was found that the organelle enlargement was about 400%. However, when hydrogenosomes suffered irreversible damage they did undergo an autophagic process and, therefore, they were destroyed, as was described previously (Benchimol, 1999). Hydrogenosomes are well known key organelles involved in the carbohydrate metabolism of *T. foetus* (Muller, 1993). Because such organelles are of low-redox potentials (Muller, 1993) and the benzimidazoles are oxygen species-generating drugs (Locatelli *et al.*, 2004), it can be inferred that the hydrogenosomes observed here might be partially due to a direct effect of the drug in the organelle.

As could be seen by video-microscopy, the mebendazole-treated cells were round-shaped, with an intense cytoplasm vacuolization (Fig. 3c). Such vacuoles, which varied in shape and size, exhibited a content resembling membranaceous material. Cytoplasmic vacuolization is often associated with an autophagic process which in turn, is associated with cell death (Mariante *et al.*, 2006). Previously published data reported the occurrence of such vacuolization processes in other microorganisms (Christensen *et al.*, 1998; Sperandio *et al.*, 2000; Wyllie & Goldstein, 2001), including *T. foetus* (Benchimol, 1999, 2001; Ribeiro *et al.*, 2002; Mariane *et al.*, 2003; Madeira-da-Costa & Benchimol, 2004), when they are under stress conditions such as drug treatments.

In comparison with the other two assayed benzimidazoles, albendazole caused just a slight alteration in the general morphology of *T. foetus* without affecting parasite motility. The ultrastructural data relating to these parasites revealed that the treatment with albendazole resulted in a significant increase in cytoplasmic vacuolization.

Altogether, the results reported here demonstrated that drugs of the benzimidazole family, especially mebendazole, are highly effective against *T. foetus*. Nevertheless, such benzimidazole efficacy could be detected in *in vitro* conditions in which oxygen was present throughout experiments favoring oxidation of the benzimidazole molecules. It would be of great interest to investigate the effects of benzimidazoles on *T. foetus* cultivated under anaerobic conditions.

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### Dedication

The authors dedicate this paper to the memory of Prof. Luiz Henrique Monteiro Leal: their supervisor and friend and a person whose spirit and enthusiasm still live with them.

### References


