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Effects of metronidazole analogues on *Giardia lamblia*: experimental infection and cell organization ☆

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ABSTRACT

The chemotherapeutic agents used for the treatment of giardiasis are often associated with adverse side effects and are refractory cases, due to the development of resistant parasites. Therefore the search for new drugs is required. We have previously reported the giardicidal effects of metronidazole (MTZ) and its analogues (MTZ-Ms, MTZ-Br, MTZ-N_3, and MTZ-I) on the trophozoites of *Giardia lamblia*. Now we evaluated the activity of some giardicidal MTZ analogues in experimental infections in gerbils and its effects on the morphology and ultrastructural organization of *Giardia*. The giardicidal activity in experimental infections showed ED_{50} values significantly lower for MTZ-I and MTZ-Br when compared to MTZ. Transmission electron microscopy was employed to approach the mechanism(s) of action of MTZ analogues upon the protozoan. MTZ analogues were more active than MTZ in changing significantly the morphology and ultrastructure of the parasite. The analogues affected parasite cell vesicle trafficking, autophagy, and triggered differentiation into cysts. These results coupled with the excellent giardicidal activity and lower toxicity demonstrate that these nitroimidazole derivates may be important therapeutic alternatives for combating giardiasis. In addition, our results suggest a therapeutic advantage in obtaining synthetic metronidazole analogues for screening of activities against other infectious agents.

1. Introduction

Giardia lamblia is a parasitic protozoan that colonizes the human intestinal tract causing a wide clinical spectrum disorder called giardiasis. The disease is a zoonosis which is considered an important public health problem in many countries worldwide. It infects about 200 million people in Asia, Africa, and Latin America (Yason and Rivera, 2007). *G. lamblia* is a major cause of diarrhea among children and travelers (Buret, 2008).

Infected individuals may be asymptomatic or present dehydration-causing diarrhea and abdominal discomfort. Giardiasis can produce chronic diarrhea, lasting for several months, which may result in malabsorption and weight loss, contributing to the increased mortality of individuals who are malnourished or immune deficient in the first 3 years of life (Buret et al., 2002; Ankarklev et al., 2010; Wensaas et al., 2010; Cotton et al., 2011). Both host and parasite factors contribute to the pathogenesis of giardiasis. Malabsorption, maldigestion, chloride hypersecretion, and increased rates of small intestinal transit are the main factors involved in the onset of diarrhea (Buret, 2008; Cotton et al., 2011).

Therefore *G. lamblia* has been implicated in the disturbance of physical (Farthing et al., 1986; Simsek et al., 2004) and cognitive (Thompson et al., 1993) development among children. It is estimated that the incidence of giardiasis in the world reached 1 billion cases, constituting one of the most common protozoan infection (Wright et al., 2003). Nevertheless, it is a neglected disease. A variety of chemotherapeutic agents have been used in the treatment of giardiasis. However, most of the drugs used display significant side effects and are contraindicated in some cases. Moreover, *Giardia* is able to develop resistance to these agents (Wright et al., 2003; Müller et al., 2000). Giardiasis was included in the 'Neglected Diseases Initiative' (Savioli et al., 2006) highlighting the need for new effective nontoxic giardicidal drugs.

The introduction of nitroheterocyclic drugs, in the 1950s, represented a new era in the treatment of bacterial and protozoan infections. Metronidazole (1- β-hydroxyethyl-2-methyl-5-nitroimidazole) is currently the most widely used drug for the treatment of infections caused by *G. lamblia*, *Entamoeba histolytica*, *Trichomonas vaginalis*, and *Blastocystis* spp. (Upcroft and Upcroft, 2001; Busatti et al., 2009; Leitsch

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et al., 2011; Mirza et al., 2011). Cases of recurrent symptoms and resistance have been documented in all these parasites (Upcroft et al., 2006; Bansal et al., 2006; Tejman-Yarden et al., 2011), encouraging the advancement of research on alternative drugs against these parasites.

Metronidazole (MTZ) analogues have been developed and tested against different microorganisms, and some of them have proved effective against *Giardia*, *Trichomonas*, and *Entamoeba histolytica* (Upcroft et al., 2006; Barbosa et al., 2006; Busatti et al., 2007).

Previously, we reported the activity of MTZ analogues against *G. lamblia* (Busatti et al., 2007). Here we evaluated the giardicidal activity of some MTZ analogues in experimentally infected gerbils and employed an ultrastructural analysis as an instrumental tool to clarify the mechanisms of action of these potential drugs upon the parasite.

2. Material and methods

2.1. Synthesis of MTZ analogues

MTZ analogues were obtained by reactions as described previously (Busatti et al., 2007). The purity of these compounds was evaluated by thin layer chromatography on silica gel plates. The structures were confirmed by spectral data analysis (RMN^{1}H and RMN^{13}C). The dose–response curves were obtained by associating axenic cultures of *Giardia* with increasing concentrations of MTZ, MTZMs, MTZN$_3$, MTZ-Br, and MTZI, ranging from 0.08 to 30 μmol/L.

2.2. Cultures and growth conditions

G. lamblia Portland strain (ATCC 30888) was used in all experiments. It was kept axenically at 37 °C in Diamond's modified TYI-S-33 medium (Keister, 1983) supplemented with heat-inactivated bovine serum at 10%. To quantify the drug's action, 1.5×10^5 trophozoites of *Giardia* were grown in culture plates of 24 wells (Nunc, Berkeley, CA, USA) in CO_2 atmosphere at 37 °C for 48 h.

2.3. Antigiardial activity in vivo

Giardicidal activity, in vivo, of MTZ analogues was assessed by determining the parasitic load of trophozoites as previously described (Belosevic et al., 1983; Araújo et al., 2008) with some modifications. The activity of MTZ analogues on *Giardia* trophozoites was evaluated in vivo using gerbils (*Meriones unguiculatus*) as an experimental model. The experiments were performed in compliance with the guidelines of the Institutional Animal Care and Committee on Ethics of Animal Experimentation (Comitê de Ética em Experimentação Animal–CETEA, national guidelines Lei 11.794, de 8 de outubro de 2008) of Universidade Federal de Minas Gerais (UFMG; protocol number 181/2008, approved on 03/04/2009). Animals aged 4–8 weeks of both sexes were used. They were divided into groups of 6 animals for each compound: a negative control group (in the absence of nitroimidazoles), a group that received the vehicle (phosphate buffered saline [PBS, pH 7.2], containing dimethyl sulfoxide [DMSO, 0.05%]); a positive control group (in the presence of metronidazole); and 5 test groups, where animals infected with *G. lamblia* were treated with 0.1 to 6.0 μmol/kg of MTZ and its analogues, MTZ-I and MTZ-Br.

For the inhibition assay, 1×10^6 *G. lamblia* trophozoites in 1 mL of PBS were inoculated in gerbils by gavage. Six days after inoculation, the animals were treated intragastrically with 1 mL of the nitroimidazoles, dissolved in PBS containing 0.05% DMSO. Two days after the treatment with drugs (8 days after inoculation), the animals were sacrificed and 18 cm of the small intestine of each animal was removed, opened longitudinally, and placed in glass tubes containing 10 mL of cold PBS for 20 min. ED_{50}, which is the dose leading to 50% parasite growth inhibition, compared to growth in the control, was determined for each compound.

2.4. Transmission electron microscopy

Parasites were fixed in 4% paraformaldehyde (Polysciences, Warrington, PA, USA), 2.5% glutaraldehyde (Polysciences), 4% sucrose in 0.1 mol/L sodium cacodylate buffer (pH 7.2) for at least 1 h, post-fixed in 1% osmium tetroxide (Polysciences) and 0.8% potassium ferricyanide in the same buffer for 40 min, dehydrated in acetone series, and embedded in Polybed resin (Polysciences). Thin sections were stained with 2% uranyl acetate for 20 min and with 1% lead citrate for 5 min and observed under a Zeiss 900 transmission electron microscope (Carl-Zeiss, Oberkochen, Germany).

The morphometric analysis of peripheral vesicles of trophozoites was performed before and after MTZ-I treatments. The area determination was made based on the limits of these organelles, using the software SIS iTEM (SIS iTEM, Palatka, FL, USA). Three vesicles per cell were selected randomly on at least 20 cells observed on ultrathin sections. The data plotted in GraphPad Prism 5.0 (GraphPad, San Diego, CA, USA) are represented as the mean ± SEM and were analyzed by Student's *t* test with a significance level of $P < 0.05$.

2.5. Statistical analysis

Each experiment was done at least 3 times in triplicate. Analysis of variance (ANOVA) was used to analyze differences between IC_{50} (dose required for 50% growth inhibition in vitro) and ED_{50} (dose required to inhibit 50% of organisms growth in vivo) values. *P* values below 0.05 were considered statistically significant.

3. Results

3.1. Antigiardial activity

The IC_{50} of MTZ-Ms (0.69 ± 0.05), MTZ-N3 (0.70 ± 0.16), MTZ-I (0.40 ± 0.03), and MTZ-Br (0.28 ± 0.04) tested presented higher giardicidal activity when compared with MTZ (1.96 ± 0.13). MTZ-Br and MTZ-I were the most active ($P < 0.001$), so they were chosen to determine the antigiardial activity in vivo.

Both the MTZ analogues MTZ-I and MTZ-Br were able to significantly ($P < 0.001$) reduce the *G. lamblia* parasite load in infected gerbils. MTZ-Br and MTZ-I had greater giardicidal activity, with ED_{50} values significantly lower than the MTZ (Table 1).

3.2. Transmission electron microscopy

Ultrastructural analysis of untreated control *G. lamblia* trophozoite fixed after 48 h of incubation in the presence of 0.05% DMSO did not induce alterations in the ultrastructure of the protozoan (Fig. 1A), indicating that this solvent concentration was nontoxic.

Trophozoites incubated with MTZ showed evident ultrastructural disorganization (Fig. 1B) characterized by the centripetal displacement of the peripheral vesicles and internalization of the cytoskeletal components of the flagella and adhesive disk into the cytoplasmic matrix.

Table 1
Activity in vivo of MTZ analogues against *G. lamblia* in gerbils.[a]

Compound	ED_{50} (μmol/kg)[b]
MTZ[c]	0.74 (0.78–0.72)
MTZ-Br	0.51 (0.53–0.49)
MTZ-I	0.38 (0.42–0.34)

[a] Results are expressed as mean ($n = 6$).
[b] Dose required to inhibit 50% of organism growth with 95% confidence limits.
[c] Positive control.

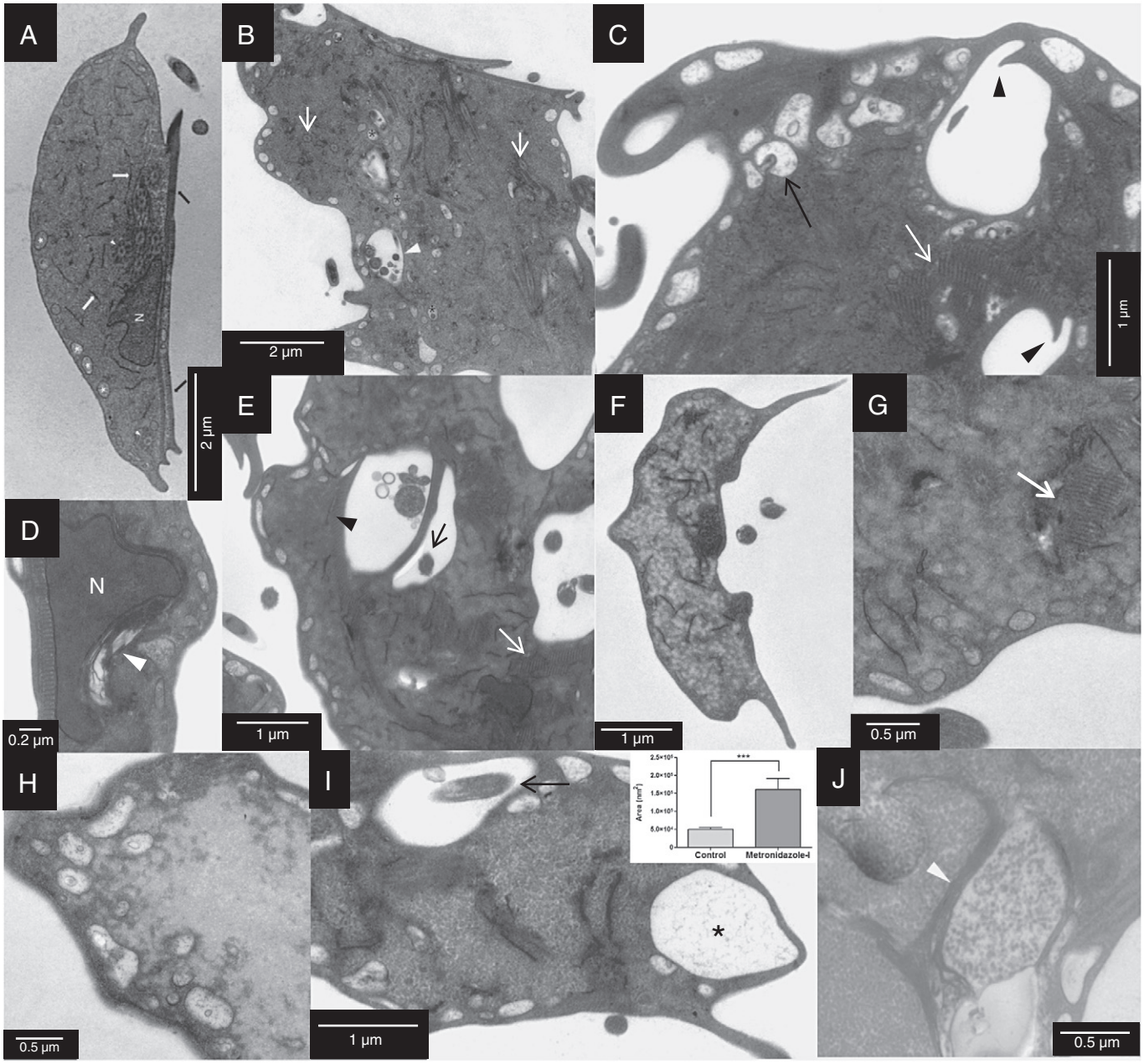

Fig. 1. (A) Cross section of *Giardia lamblia* trophozoite incubated with 0.05% DMSO showing characteristic ultrastructure, with the presence of peripheral vesicles (*), endoplasmic reticulum cisternae (white arrows), nucleus (N), adhesive disk (black arrows), and axonemes of the flagella (arrowheads) within the homogeneously electron-dense cytoplasm. (B) *G. lamblia* trophozoites incubated with 0.85 µmol/L of metronidazole for 48 h, with ultrastructural disorganization, centripetal displacement of peripheral vesicles (*), and flagellar internalization (arrowhead) as well as numerous cytoplasmic axonemes (arrows). (C) Trophozoites incubated for 48 h in the presence of 0.51 µmol/L of metronidazole-N3 displaying peripheral vesicle dilatation and interdigitation (black arrow), as well as concave regions of adhesion disk engulfment areas (arrowheads) and disassembly of its cytoskeleton components (white arrow). (D) Trophozoites incubated for 48 h in the presence of 0.51 µmol/L of metronidazole-N3 displaying the formation of extensive concentric arrays of endoplasmic reticulum cisternae (arrowhead) circumscribing the nuclei (N). (E) Cells incubated for 48 h in the presence of 0.48 mmol/L of metronidazole-MS showed marked disorganization of the cellular architecture of trophozoites and internalization of the flagella (black arrow), adhesive disk (arrowhead), and its components' disassembly (white arrow). (F) We also observed a lower cytoplasmic electrondensity and the absence of glycogen granules. (G) and (H) *G. lamblia* trophozoites treated with 0.32 µmol/L of metronidazole-Br for 48 h. We observed internalization and disaggregation of adhesive disk cytoskeleton components (G, arrow), heterogeneous cytoplasmic granulosity, and electrondensity properties as many parasites displayed remarkable cytoplasmic extraction (H), resulting in altered cell architecture. (I) and (J) *G. lamblia* trophozoites treated with 0.26 µmol/L of metronidazole-I displayed remarkably swollen vesicles (I, *), which may be involved in the internalization of the flagella (arrow). Morphometry of the peripheral vesicles revealed that the average area in the 0.26 µmol/L metronidazole-I–treated images was significantly (***, $P < 0.001$) enhanced (I, inset). Several cells presented autophagic process (J), characterized by concentric membranes (arrowhead) surrounding the cytoplasmic material presumably in degradation.

Parasites incubated with MTZ-N_3 displayed large areas of adhesive disk internalization and peripheral vesicle interdigitation (Fig. 1C). Several cells presented multilayered membranes stacked in the perinuclear area (Fig. 1D).

Altered parasite cell architecture and adhesive disk internalization were also found in trophozoites incubated with MTZ-Ms (Fig. 1E). Lower cytoplasmic electrodensity and absence of glycogen granules were also observed (Fig. 1F). Heterogeneous cytoplasmic granulosity and electrondensity properties were observed in *Giardia* trophozoites treated with 0.32 µmol/L metronidazole-Br for 48 h (Fig. 1G), as well as internalization of adhesive disk cytoskeleton components (arrowhead). Many parasites displayed remarkable cytoplasmic extraction (Fig. 1H), resulting in altered cell architecture.

Parasites grown in the presence of MTZ-I presented remarkably large vesicles, which may be involved in the internalization of the flagella (Fig. 1I). Morphometric analysis of 0.26 μmol/L of MTZ-I–treated parasites revealed 3-fold ($P < 0.001$) enlargement of sectioned peripheral vesicle areas as compared to controls (Fig. I, insert). Autophagic vacuole formation surrounding the cytoplasmic material presumably in degradation was also observed (Fig. 1J).

4. Discussion

Despite its significant morbidity causing considerable socioeconomic impact, especially in underdeveloped countries, giardiasis is largely overlooked.

Besides sanitation, disease control could be accomplished by vaccination (Olson et al., 2000) and with new drugs to avoid the resistance phenomenon (Argüello-García et al., 2009; Leitsch et al., 2011). MTZ analogues are efficient giardicidal compounds and could become an alternative for the treatment of giardiasis (Upcroft et al., 2006; Busatti et al., 2007). Thus, we evaluated the giardicidal activity of some MTZ analogues in experimentally infected gerbils and its effects on the morphology and ultrastructural organization of *Giardia*.

All analogues tested displayed higher anti-*Giardia* activity than the MTZ. The analogues were able to significantly reduce the parasite load of gerbils infected with *G. lamblia*. In an electrochemical study, these analogues showed greater reduction potential for MTZ-Br followed by MTZ-I and MTZ (Cavalcanti et al., 2004). These results highlight the reduction potential as a chemical giardicidal property relevant to the activity of these compounds. Reduction leads to the formation of toxic nitro radicals, which are responsible for the destabilization of DNA and the production of other toxic radicals that react with essential cellular components, interfering with cellular metabolism (Edwards, 1993; Leitsch et al., 2011).

The transmission electron microscopy revealed that trophozoites of *G. lamblia* treated with MTZ and its analogues showed a marked change in cellular architecture with evident ultrastructural disorganization. Trophozoites treated with the analogues showed changes similar to those observed in parasites treated with MTZ, such as centripetal displacement of the peripheral vesicles, internalization of the flagella and cytoskeletal components of the adhesive disk, vacuolization, and dilation and extraction of peripheral cytoplasmic vesicles, indicating that the mechanisms of action of these compounds caused similar metabolic changes in the parasite.

Antiparasitic drugs may affect the protozoa endocytic (Bernardes et al., 2000) and/or autophagic pathways (Vannier-Santos et al., 1999; Vannier-Santos and de Castro, 2009). The formation of concentric membranes and myelin figures is indicative of autophagic processes; these changes were particularly observed in trophozoites treated with MTZ-I analogues. The engulfment of digit-form processes between adjacent peripheral vesicles might characterize microautophagy triggering as vacuolar tubular invaginations may be involved in autophagy (Müller et al., 2007). Interestingly, the *Giardia* lysosomal functions may be performed by endoplasmic reticulum–like tubulovesicular compartments (Abodeely et al., 2009). The formation of such concentric cisternae in the perinuclear area may be related to the endoplasmic reticulum nature of the nuclear envelop. In this regard, it is noteworthy that herpes simplex virus 1–induced autophagy may be associated with the formation of concentric cisternae from the macrophage nuclear envelope (English et al., 2009). The internalization of the flagella and adhesion disk components is presumably associated with parasite encystment due to the drug-induced stress (Maia et al., 2008).

In previous studies, we observed that the analogues of MTZ were not cytotoxic in vitro to human cells in a concentration range from 0.2 to 6.4 μmol/L (Busatti et al., 2007). These results coupled with the excellent giardicidal activity in vivo and in vitro demonstrate that these nitroimidazole derivates may be important therapeutic alternatives to combat giardiasis. Furthermore, the present results suggest a therapeutic advantage with obtaining synthetic analogues of MTZ for screening of activities against other infectious agents.

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