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The effect of metronidazole on the cell cycle and DNA in metronidazole-susceptible and -resistant *Giardia* cell lines

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ABSTRACT

Metronidazole (MTZ) is used as the drug of choice to treat *Giardia* infections. It is believed that the prodrug is transformed intracellularly into toxic intermediates that interact with cellular components, leading to cell death. The present study aimed to describe the effects of MTZ treatment on DNA and cell cycle progression in MTZ-sensitive and *in vitro*-derived MTZ-resistant cell lines. Detection of the phosphorylated form of histone H2A in cell nuclei together with electrophoresis of genomic DNA, flow cytometry analysis and incubation of cells with other drugs (albendazole or neomycin) demonstrated that DNA damage in MTZ-treated cells is clearly conditioned by the presence of this drug. The flow cytometry analysis and a BrdU labeling assay showed that the sublethal drug concentration affects the replication phase of the cell cycle. Cells incubated with lethal drug concentration exhibit unchanged DNA profile, only about 50% of cells are positive for γH2A and lose an ability to attach to a surface after few hours of incubation. It is likely that the early reaction of cells to lethal concentration of MTZ is not primarily initiated by the reaction to DNA damage but rather by the immediate interaction of MTZ with biomolecules where activated MTZ is generated. Interestingly, in MTZ-resistant lines incubated in the presence of the drug, about 40% of cells remain permanently positive for γH2A without any effects on the cell cycle progression suggesting that DNA damage caused by MTZ treatment persists in these cells. Accelerated mutagenesis caused by MTZ-induced DNA damage may therefore be a new factor contributing to the development of natural resistance.

1. Introduction

Giardia intestinalis is a unicellular binucleated parasite of the small intestine of mammals with a worldwide distribution [1]. Human giardiasis is characterized by diarrhea and consequent malabsorption, which, in its chronic form, may lead to serious health problems [2]. Metronidazole (MTZ), the first 5-nitroimidazole derivative introduced as an antimicrobial agent against a variety of prokaryotic and eukaryotic anaerobes and microaerophiles more than 50 years ago, is the drug of choice for giardiasis and remains the most commonly prescribed medication to treat *Giardia* infection. MTZ is believed to be a prodrug that undergoes intracellular enzymatic activation [3]. It is widely accepted that reduction of the 5-nitro group of MTZ by electrons derived from intracellular donor(s) yields toxic intermediates, specifically a nitro anion radical and nitroso derivatives, that are presumed to cause DNA damage [4]. Previous electrochemical studies have demonstrated that *in vitro*, the radical formed *via* reduction of the nitro group interacts with DNA bases [5]. Drug target studies conducted *in vitro* have shown that oxidative damage to DNA resulting in strand breaks occurs in the presence of reduced MTZ [4]. However, a more complex response appears to occur *in vivo*, as suggested by recent findings in anaerobic protists [6–8]. *In vivo* detection of nitro radical anions has only been described in *Trichomonas* [9] and the electron paramagnetic resonance signal from metronidazole nitro anion radical could not be demonstrated in *Giardia* [10].

At least four enzymes can reduce MTZ in *Giardia* cells. In addition to pyruvate:ferredoxin oxidoreductase (PFOR), three other reductases, specifically thioredoxin reductase (GlTrxR) and nitroreductases 1 and 2 (GlNR1 and GlNR2), were recently identified as drug targets [6,7,11]. Interestingly, *Giardia* GlNR2 appears to detoxify MTZ by converting it into a non-toxic end product *via* complete reduction of the drug [7]. Results from a recent proteomic analysis suggest that MTZ binds to several unrelated *Giardia* proteins (*e.g.*, elongation factor-1γ and beta-giardin), among which only thioredoxin reductase is involved in redox processes [6]. This finding indicates that the drug may affect different metabolic pathways

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in *Giardia* and that DNA damage may be secondary to the drug's direct attack on specific protein(s). However, to date, there is only one evidence that MTZ treatment induces DNA damage *in vivo*, *i.e.*, in intact *Giardia* cells. Caspase-independent programmed cell death has been described in *Giardia* cells treated with MTZ [12,13], but the mechanism of death is unknown; TUNEL assays used to show DNA fragmentation may detect both apoptosis- and necrosis-derived DNA strand breaks, and transmission electron microscopy did not reveal any changes in nuclear ultrastructure in cells exposed to high concentrations of MTZ. Instead, swelling of peripheral vesicles, the presence of large multi-membrane vesicles within the cytoplasm, and damage to the plasma membrane were observed [14].

When mammalian cells are exposed to a DNA damaging agent, a signaling network termed the DNA damage response (DDR) is activated. DDR recognizes DNA lesions and initiates either repair of the damaged DNA (coordinated with the cell cycle) or permanent cell cycle arrest or cell death when a lesion is not repairable [15]. In *Giardia*, DDR is poorly understood, and only a few studies have focused on this issue. In *Giardia* cells exposed to the DNA polymerase inhibitor aphidicolin, histone H2A was shown to be phosphorylated to the γH2A variant using specific antibodies [16]. Posttranslational phosphorylation of histone H2A is a common marker of DNA damage that occurs in all eukaryotes sharing the SQ motif in the C-terminal portion of the protein [17]. However, a study of the *Giardia* kinome revealed that the parasite lacks key components of the DNA damage signaling network, specifically orthologs of apical kinases and checkpoint kinases [18].

Although MTZ is the most commonly used drug to cure giardiasis, treatment fails in approximately 20% cases with recurrence up to 90% suggesting resistance of the parasite to the drug. And surprisingly, clinical isolates naturally resistant to MTZ have not been described despite the fact that several MTZ resistant cell lines have been successfully generated *in vitro* [19]. MTZ resistance in *Giardia* was historically linked to decreased activity of PFOR, the enzyme responsible for MTZ activation *via* transport of electrons from a reduced form of ferredoxin during anaerobic glycolysis [4]. The existence of resistant cell lines with fully functional PFOR [20], other resistant lines with impaired activity of thioredoxin reductase [6] and *Giardia* cells less susceptible to MTZ due to overexpression of nitroreductase GlNR2, a MTZ-inactivating enzyme [7] indicates multifaceted mechanism(s) of resistance in *Giardia*.

The present work aims to describe the effects of MTZ treatment on DNA and cell cycle progression in both MTZ-sensitive and -resistant *Giardia* cell lines and thus to contribute to the understanding of the cytotoxic reaction to this most commonly used drug.

2. Materials and methods

2.1. Giardia cultures

The following isolates and clones were used: the genome project clone C6 of the WB isolate (ATCC 50803) and the isolates BRIS/83/HEPU/106 (106) and BRIS/87/HEPU/713 (713) with their respective isogenic metronidazole-resistant lines 713-M3 and 106-2ID10 exhibiting decreased PFOR activity [20]. The cells were grown in filter-sterilized TYI-S-33 medium supplemented with bovine bile in glass screw cap tubes at 37 °C. Resistant isolates were maintained in the presence of 5 μM MTZ. The cells were subcultured every 3–4 days. Stock solutions of MTZ (Fluka, M3761), albendazole (ALB) (Sigma, A4673) and neomycin (NEO) (ZellBio GmbH, G-418-5) were prepared as follows: A stock solution of MTZ (5 mg/ml in H_2O) was sterilized by autoclaving and maintained at 4 °C; a stock solution of ALB (750 μg/ml in dimethyl sulfoxide) was stored at 4 °C and a filter-sterilized stock solution of NEO (100 mg/ml in H_2O) was kept at −20 °C. The stock solutions were diluted into medium immediately before use. Final concentrations of 1.25, 2.5, 5, 10, 20, 50 and 100 μM MTZ, 5 μg/ml ALB or 600 μg/ml NEO were used. For all experiments, cells were grown in either screw cap tubes and in 1.5 ml Eppendorf tubes or in 6-well and 96-well plates (flat bottom) under anaerobic conditions using the AnaeroGen system (Oxoid, AN0035). For growth curves, 1×10^4 cells per ml were seeded into 96-well microplates (in a volume of 200 μl per well). At each time point, the cells were fixed with 2 μl of a 36% formaldehyde solution, resuspended by pipetting and counted using a Burker chamber.

2.2. Flow cytometry

Flow cytometry studies were performed as previously described [21] with significant modifications. Cells grown in 1.5 ml Eppendorf tubes (approx. 10^5 cells in total) were chilled on ice for 20 min and centrifuged (1000 × *g*, 10 min, 4 °C). Then, the medium was replaced, and the cells were fixed with 1 ml of ice cold 70% ethanol and stored at 4 °C. On the day of measurement, the cells were centrifuged (3500 × *g*, 5 min); washed with PBS, pH 7.2; centrifuged again; resuspended in 500 μl of PBS containing 2.5 μl of RNase A corresponding to 50 μg/ml (Fermentas, EN0531); and incubated for 30 min at 37 °C. Then, the cells were centrifuged and resuspended in 500 μl of filter-sterilized staining solution (10 mM Tris/10 mM $MgCl_2$/PBS) containing propidium iodide at a final concentration of 2 μg/ml (Sigma, P4864). All samples were measured at approximately the same time using a BD FACSCanto™ II instrument (BD). The events were recorded at a low speed of approximately 200–400 events/s, and at least 10,000 events were recorded for each histogram. The data were analyzed using FACSDiva software, and high-resolution graphs were generated according to previously published processes [22].

2.3. Immunofluorescence microscopy

The labeling of adherent cells to detect γH2A was performed as previously described [16] with minor modifications. Double labeling was performed as follows. Fixed cells were incubated with a mixture of two primary antibodies in 2% bovine serum albumin/0.1% Triton X-100/PBS, pH 7.4, diluted 1:250 for the polyclonal antibody against phosphorylated Ser139 in histone H2AX (Active Motif, 39117) and 1:500 for the monoclonal antibody against acetylated α-tubulin (Sigma, T6793). After a series of PBS washes, a mixture of the corresponding secondary antibodies was used, including a FITC-conjugated goat anti-rabbit IgG antibody (Sigma, F0382) diluted 1:250 and a Cy3-conjugated donkey anti-mouse IgG antibody (Jackson ImmunoResearch, 715-165-150) diluted 1:500. Finally, the cells were mounted in Vectashield with DAPI (Vector laboratories, H-1200). The positivity of nuclei for γH2A was determined for the WBc6 clone (1) in cells treated with 1.25, 2.5, 5 and 10 μM MTZ for 24 h; (2) in cells incubated at a constant concentration of 5 μM MTZ for 12 and 24 h; and (3) in cells incubated in 5 μM MTZ for 24 h and then with fresh MTZ-free medium for another 24 h. Isolates 106 and 713 and their corresponding MTZ-resistant lines were grown in the presence of 5 μM MTZ for 12 or 24 h. For all experiments, the same procedure was applied to untreated cells that were used as controls. Positivity for γH2A in one or both nuclei in a cell was determined to be positive for γH2A in all graphs. For each time point, 200 cells were examined. An Olympus BX51 fluorescence microscope was used for all observations.

2.4. BrdU labeling assay

Replicating cells were distinguished from non-replicating cells using an immunofluorescence assay to detect BrdU incorporated