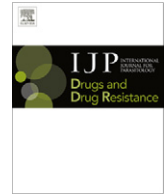




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Brief Report

Nitroimidazole drugs vary in their mode of action in the human parasite *Giardia lamblia*

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ABSTRACT

Giardia lamblia (syn. *duodenalis*, *intestinalis*) is a globally occurring micro-aerophilic human parasite that causes gastrointestinal disease. Standard treatment of *G. lamblia* infections is based on the 5-nitroimidazole drugs metronidazole and tinidazole. In two other micro-aerophilic parasites, *Entamoeba histolytica* and *Trichomonas vaginalis*, 5-nitroimidazole drugs bind to proteins involved in the thioredoxin-mediated redox network and disrupt the redox equilibrium by inhibiting thioredoxin reductase and depleting intracellular thiol pools. The major aim of this study was to assess whether nitroimidazoles exert a similar toxic effect on *G. lamblia* physiology.

The 5-nitroimidazoles metronidazole and tinidazole were found to bind to the same subset of proteins including thioredoxin reductase. However, in contrast to *E. histolytica* and *T. vaginalis*, none of the other proteins bound are candidates for being involved in the thioredoxin-mediated redox network. Translation elongation factor EF-1 γ , an essential factor in protein synthesis, was widely degraded upon treatment with 5-nitroimidazoles. 2-Nitroimidazole (azomycin) and the 5-nitroimidazole ronidazole did not bind to any *G. lamblia* proteins, which is in contrast to previous findings in *E. histolytica* and *T. vaginalis*. All nitroimidazoles tested reduced intracellular thiol pools in *G. lamblia*, but metronidazole, also in contrast to the situation in the other two parasites, had the slightest effect. Taken together, our results suggest that nitroimidazole drugs affect *G. lamblia* in a fundamentally different way than *E. histolytica* and *T. vaginalis*.

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1. Introduction

Giardia lamblia (syn. *duodenalis*, *intestinalis*) is considered to be the most common protozoan intestinal parasite worldwide (Buret, 2008). It causes giardiasis, a disease with symptoms such as diarrhea, nausea and failure to thrive in infected children. Treatment of giardiasis is mainly based on metronidazole (Tejman-Yarden and Eckman, 2011), a 5-nitroimidazole drug which is exclusively toxic to microaerophilic and anaerobic microorganisms (Samuelson, 1999). Alternatively, another 5-nitroimidazole drug, tinidazole, is often prescribed (Fung and Doan, 2005).

The selective toxicity of 5-nitroimidazoles to microaerophiles and anaerobes has been ascribed to the existence of factors in these organisms that can transfer electrons to the nitro group and, therefore, activate the prodrug metronidazole to its active state (Müller,

1983). Further, since the single electron transfer product, the nitro-radical anion, is easily re-oxidized by oxygen to the parent compound (Pervez-Reyes et al., 1980) effective doses of activated drug are only achieved in organisms with very low intracellular oxygen concentrations. It is still not fully understood whether the nitro radical anion state or a further reduced state, e.g. the nitrosoimidazole state, is the actual agent (Moreno and Docampo, 1985). There is certain evidence that the latter could be the case (West et al., 1982; Wislocki et al., 1984). In *G. lamblia*, several factors have been suggested to reduce 5-nitroimidazoles, including ferredoxin, which in turn receives electrons from pyruvate:ferredoxin oxidoreductase (Townson et al., 1996), nitroreductase 1 (Nililius et al., 2011), and thioredoxin reductase (Leitsch et al., 2011).

Comparably little is known about the mode of action of 5-nitroimidazoles in *G. lamblia*. In general, these drugs are considered to damage DNA (Edwards, 1993) but it has as yet not been conclusively demonstrated that this damage is caused by direct attack of 5-nitroimidazoles on DNA rather than by secondary events (LaRusso et al., 1978). Indeed, it was recently suggested that metronidazole induces programmed cell death in *G. lamblia* which also includes fragmentation of DNA (Ghosh et al., 2009; Bagchi et al.,

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2012). Further, ultrastructural studies revealed damage to the dorsal surface and the lateral flange upon treatment with metronidazole (Campanati and Monteiro-Real, 2002; Müller et al., 2006). Also damage to the ventral disk was observed (Oxberry et al., 1994) but this effect was not confirmed in other studies (Campanati and Monteiro-Real, 2002; Müller et al., 2006).

In our previous work on metronidazole action in *Entamoeba histolytica* (Leitsch et al., 2007) and *Trichomonas vaginalis* (Leitsch et al., 2009) we performed proteomic analyses with nitroimidazole-treated parasites and showed that metronidazole forms covalent adducts with a defined subset of proteins, including thioredoxin reductase and proteins that are known or likely to depend on thioredoxin-mediated reduction. Thioredoxin reductase activity was strongly diminished after nitroimidazole treatment, resulting in impaired removal of hydrogen peroxide by peroxidases (Leitsch et al., 2009). Furthermore, nitroimidazoles were shown to deplete intracellular thiol pools (Leitsch et al., 2007, 2009). Taken together, our results suggested that nitroimidazoles disrupt the redox system in these parasites.

In order to shed more light on metronidazole action in *G. lamblia* and to find further evidence for our claim that nitroimidazoles strike at the cellular redox system in microaerophilic parasites, we performed an analogous study in *G. lamblia* using a variety of nitroimidazole drugs, including metronidazole, tinidazole, ronidazole and 2-nitroimidazole (azomycin).

2. Materials and methods

2.1. Nitroimidazoles

Metronidazole, ronidazole, tinidazole, and 2-nitroimidazole (azomycin) were purchased from Sigma–Aldrich.

2.2. Cell culture

G. lamblia BRIS/83/HEPU/106 (referred to as 106) was grown in completely filled and tightly closed 25 ml polystyrene culture flasks (Falcon, Becton–Dickinson) in modified TYI-S-33 medium containing bovine bile (Keister, 1983).

2.3. Analysis by two-dimensional gel electrophoresis (2DE)

G. lamblia cultures ($2\text{--}5 \times 10^7$ cells) were harvested at RT by centrifugation at 750g (5 min). Cell pellets were subsequently washed in two centrifugation steps (750g at RT, 5 min) in $1 \times$ PBS. Cells were resuspended in 2 ml of 10% trichloroacetic acid, 20% ultrapure water and 70% acetone, and incubated at -20°C for at least 1 h. Afterwards, the samples were centrifuged at 20,000g at 4°C and washed twice by in 90% acetone followed by centrifugation at 20,000g (at 4°C for 20 min). Samples were resuspended in an appropriate amount of sample buffer (7 M urea, 2 M thiourea, 1% dithiothreitol, 1% ampholytes pH 3–10) and insoluble material was removed by centrifugation at 20,000g (at 20°C for 20 min). Two-dimensional gel electrophoresis (2DE) was performed as described (Leitsch et al., 2005). For isoelectric focussing in 17 cm IPG strips, 500 μg of protein were loaded. The second dimension was run over 20×20 cm gels in a Protean II xi cell (Bio-Rad). Gels were Coomassie-stained and images were evaluated using Melanie™ 4 software (Genebio).

2.4. Identification of proteins by mass spectrometric analysis

Protein identification was performed as described recently (Leitsch et al., 2011). Excised protein spots were digested with 0.02 mg/ml trypsin (modified, sequencing grade, Roche).

Subsequently, acidified peptides were spotted on a Bruker Anchor-Chip™ target with matrix ((α)-cyano-4-hydroxy-cinnamic acid) 0.5 mg/ml in 90% acetonitrile, 0.1% trifluoroacetic acid (TFA). Samples were washed with 0.5% TFA and re-crystallized in 6:3:1 ethanol:acetone:10 mM ammonium phosphate in 0.1% TFA. Mass spectrometry (MS) and tandem mass spectrometry (MS/MS) data were obtained using a Bruker Daltonics Ultraflex III, MALDI TOF/TOF mass spectrometer. Mascot software (2.2.02) was used to search against the NCBI nr database with mass tolerances of 0.4 Da for MS and 0.8 Da for MS/MS.

2.5. Measurement of non-protein thiol pools

Cultures were incubated with the concentrations of nitroimidazoles indicated for 2 h or left untreated. After harvest, pellets were washed once in $1 \times$ PBS. Pellets were resuspended in 20 mM EDTA and cells were lysed by addition of 0.1% Triton X-100. Insoluble material was removed by centrifugation at 20,000g for 10 min. Protein concentrations in the supernatants were determined by Bradford assay (Bio-Rad). Subsequently, proteins were removed by precipitation in 5% TCA at room temperature, followed by centrifugation at 20,000g for 5 min. To the supernatants the double volume each of 0.4 M Tris/HCl pH 8.9 and 1.7 μl 100 mM Ellman's reagent (DTNB) were added per ml reaction mixture. Reduction of DTNB was measured at OD₄₁₂ in a Perkin Elmer Lambda 25 UV–Vis spectrometer ($\Delta\epsilon_{412} = 13.6 \text{ mM}^{-1} \text{ cm}^{-1}$).

3. Results and discussion

3.1. Binding of metronidazole and tinidazole to a small subset of proteins including thioredoxin reductase

In analogy to our previous work on metronidazole action in *E. histolytica* (Leitsch et al., 2007) and *T. vaginalis* (Leitsch et al., 2009), two-dimensional gel electrophoresis (2DE) was performed in order to identify *G. lamblia* proteins that are bound by metronidazole and other nitroimidazoles (Fig. 1). *G. lamblia* strain 106

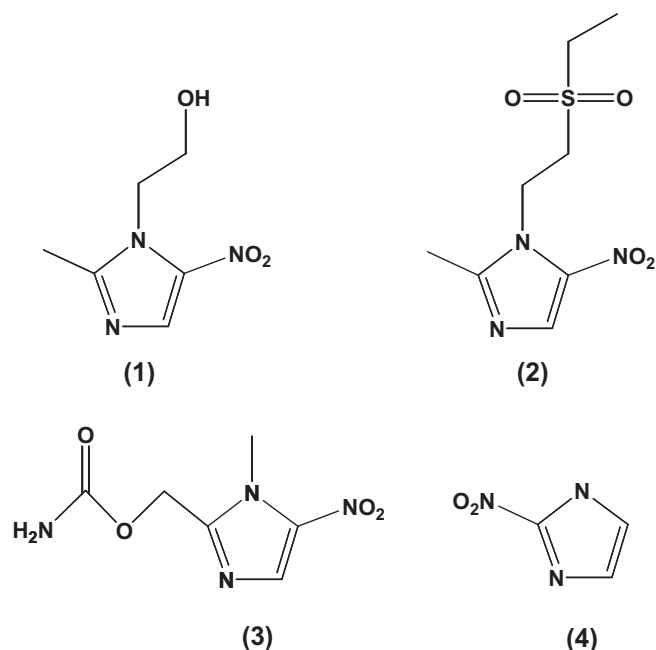


Fig. 1. The nitroimidazoles used in this study: (1) Metronidazole; (2) tinidazole; (3) ronidazole; (4) 2-nitroimidazole (azomycin).

cultures were treated for 2 h with two different concentrations of metronidazole (10 μ M, 50 μ M), followed by harvest and preparation of lysates for 2DE. In total, nine protein spots were found to be shifted to a more basic pI (Fig. 2A) as typically observed with nitroimidazole-protein adducts (Leitsch et al., 2007, 2009). Shifting of proteins in terms of pI depended on the concentration of metronidazole applied, the shifted proportions of all proteins bound being smaller at 10 μ M as compared to 50 μ M metronidazole (Fig. 2B). With two protein spots, 6 and 7 (Table 1), clearly-defined adducts could only be observed if 10 μ M metronidazole were applied (Fig. 2B), whereas treatment with 50 μ M metronidazole obviously induced degradation of the protein. In order to rule out that the observed shifts were caused by apoptotic or necrotic processes, nitroimidazole binding *G. lamblia* cultures were exposed to other nitroimidazoles (Fig. 1) which had been shown to have a similar mode of action as metronidazole in *E. histolytica* (Leitsch et al., 2007) and *T. vaginalis* (Leitsch et al., 2009). As observed before in *E. histolytica* and *T. vaginalis*, tinidazole bound the same proteins as metronidazole (Fig. 2B) but caused smaller shifts in pI due to its different charge as compared to metronidazole (Leitsch et al., 2007). Surprisingly, another 5-nitroimidazole, ronidazole (Fig. 1), only induced the degradation of EF-1 γ but did not lead to any discernible protein shifts (not shown) although it is more toxic to *G. lamblia* than any other nitroimidazole used in this study (Boreham et al., 1985). It is important to note that in *E. histolytica* ronidazole readily forms adducts with proteins (unpublished data), ruling out that the 2DE procedure itself resolves ronidazole adducts with proteins. Equally surprising, treatment with 2-nitroimidazole (azomycin) did not lead to any protein shifts (not shown) although it had been found to bind to proteins in *E. histolytica* (Leitsch et al., 2007) and in *T. vaginalis* (unpublished data). Nevertheless, 2-nitroimidazole was strongly toxic to *G. lamblia* and led to total immotility and detachment of most of the cells from the bottom of the culture flasks when being applied at 50 μ M for 2 h. Also control samples treated with the nitrofuran drug furazolidone (50 μ M for 2 h) and the benzimidazole drug albendazole (20 μ M for 3 h) did not display any discernible adduct formation (data not shown), indicating that the protein shifts observed with metronidazole and tinidazole do not reflect any apoptotic or necrotic changes but,

rather, direct binding of activated metabolites of these drugs to proteins.

Protein spots were isolated for mass spectrometric identification by peptide mass fingerprinting. The nine spots contained eight different proteins (Table 1), including thioredoxin reductase. However, with the exception of thioredoxin reductase none of the proteins identified have been reported to be involved in the thioredoxin-mediated redox system as is the case in *E. histolytica* (Leitsch et al., 2007) and *T. vaginalis* (Leitsch et al., 2009). Amongst the proteins bound were two structural proteins (giardins) and a *Giardia* trophozoite antigen (GTA-2) with unknown function. Further, an alcohol dehydrogenase with a coenzyme A acylating aldehyde dehydrogenase (ACDH) domain, pyruvate phosphate dikinase (PPDK), a branched-chain amino acid transferase, and elongation factor-1 γ (EF-1 γ), which localized to two distinct protein spots, were identified. To the best of our knowledge, none of these proteins have ever been suggested to interact with thioredoxin, neither in *G. lamblia*, nor in any other organism. It is interesting to note, however, that co-purification experiments suggest that PPDK is associated with pyruvate:ferredoxin oxidoreductase (PFOR) (Anita Burgess, unpublished results), an enzyme known to reduce nitroimidazoles via ferredoxin (Townson et al., 1996; Leitsch et al., 2011). In addition, GTA-2 and the herein identified alcohol dehydrogenase localize in native polyacrylamide gels together with PFOR (Anita Burgess, unpublished results). These observations imply that PPDK, GTA-2, and alcohol dehydrogenase might be targets of nitroimidazoles because they are closely associated with PFOR, a source of reactive nitroimidazole metabolites (Townson et al., 1996; Leitsch et al., 2011).

Unfortunately, our attempts to identify a thioredoxin which is reduced by *G. lamblia* thioredoxin reductase have so far been unsuccessful (Leitsch et al., 2011). Thus, we could not measure in the present study whether nitroimidazole treatment has a detrimental effect on thioredoxin reductase activity, as previously done in *E. histolytica* and *T. vaginalis* (Leitsch et al., 2007, 2009). A highly probable drug target of metronidazole and other 5-nitroimidazoles, however, is EF-1 γ , having an essential function in protein synthesis. Whether nitroimidazole binding to the other identified proteins contributes to the toxicity of metronidazole and tinidazole,

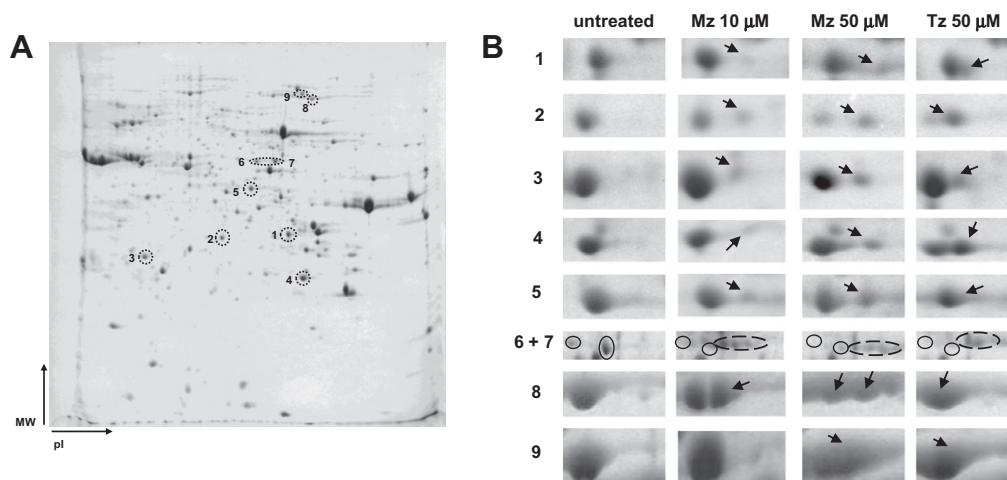


Fig. 2. (A) a 2D-gel (20 × 20 cm, pH 5–8) showing all proteins bound by metronidazole and tinidazole. (B) close-up of the affected proteins in untreated cells, and in cells either treated with 10 μ M metronidazole (Mz), 50 μ M metronidazole, or 50 μ M tinidazole (Tz) for 2 h. Covalent adducts of metronidazole and tinidazole are visible as newly appearing protein spots containing the affected protein shifted to a more basic pI (to the right). Shifted portions of proteins are indicated by arrows. In the case of proteins 6 and 7, the original spots are indicated by continuous circles and shifted portions by broken circles. Some of the protein shifts with tinidazole (proteins 5, 8, and 9) are so narrow that no new distinct spots containing tinidazole-bound protein are discernible. Instead, the original spots are extended to a more basic pI. The numbers refer to the following proteins: (1) thioredoxin reductase; (2) alpha-11 giardin; (3) β -giardin; (4) *Giardia* trophozoite antigen 2; (5) branched-chain amino acid transferase; (6) and (7) elongation factor-1 γ ; (8) pyruvate phosphate dikinase; (9) alcohol dehydrogenase. For details see Table 1.

Table 1
The eight proteins bound by metronidazole and tinidazole.

Spot	Protein	MW (Da)	Theoret. pI	Accession No.	Mowse score ^b
1	Thioredoxin reductase ^a	33,869	6.16	XP_001707168	166
2	Alpha-11 giardin	35,011	5.74	XP_001705101	522
3	β -Giardin	30,877	5.25	XP_001705425	634
4	<i>Giardia</i> trophozoite antigen 2	26,569	6.95	XP_001709922	271
5	Branched-chain amino acid transferase	39,065	6.14	XP_001706262	207
6 + 7	Elongation factor-1 γ	45,217	6.05	XP_001707321	320
8	Pyruvate phosphate dikinase	97,659	6.31	XP_001705572	416
9	Alcohol dehydrogenase	97,134	6.31	XP_001710238	522

^a Already published in Leitsch et al. (2011).

^b Mascot protein mowse scores.

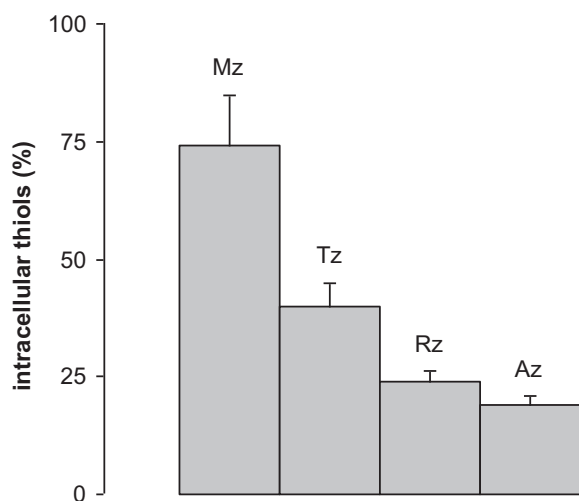


Fig. 3. Non-protein thiol content in nitroimidazole-treated cells as compared to untreated controls (100%). Mz, metronidazole; Tz, tinidazole; Rz, ronidazole; Az, 2-nitroimidazole (azomycin). All nitroimidazoles were applied at a concentration of 50 μ M for 2 h. All values were determined in at least three independent experiments each. Error bars represent SEM.

remains to be determined by application of appropriate enzyme assays. Our previous results with *T. vaginalis* (Leitsch et al., 2009) and *E. histolytica* (Leitsch et al., 2007) suggest that nitroimidazole binding only inhibits some enzyme activities, e.g. thioredoxin reductase, whereas other enzymes, e.g. thioredoxin peroxidase, enolase and malate dehydrogenase in *T. vaginalis*, are not affected (Leitsch et al., 2009).

3.2. Nitroimidazoles decrease the intracellular thiol pools of *G. lamblia*

Since nitroimidazoles compromise the redox equilibrium in *E. histolytica* (Leitsch et al., 2007) and *T. vaginalis* (Leitsch et al., 2009) by strongly decreasing non-protein thiol levels, we wanted to know whether nitroimidazole treatment leads to a similar effect in *G. lamblia*. To this end, cultures of *G. lamblia* 106 were treated with either 50 μ M of metronidazole, tinidazole, ronidazole, or 2-nitroimidazole for 2 h. Subsequently, reduced thiol pools were determined with Ellman's reagent (Leitsch et al., 2007, 2009) and compared to untreated controls (Fig. 3). The non-protein thiol content of untreated *G. lamblia* 106 was 8.8 ± 3.7 nmol (mg protein)⁻¹.

All nitroimidazoles tested decreased non-protein thiol levels. However, in stark contrast to *E. histolytica* (Leitsch et al., 2007) and *T. vaginalis* (Leitsch et al., 2009), treatment with 2-nitroimidazole had the strongest effect, whereas metronidazole reduced thiol levels by only 25% (Fig. 3).

4. Conclusion

Taken together, our observations strongly suggest that nitroimidazoles are differently metabolized in *G. lamblia* as compared to the other two parasites. Of the nitroimidazoles tested, only metronidazole and tinidazole formed adducts with proteins (Fig. 2). Treatment with ronidazole only induced the degradation of EF-1 γ , and 2-nitroimidazole had no discernible effect on the 2D-profile of treated *G. lamblia* at all. Further, nitroimidazoles affected intracellular thiol pools in *G. lamblia* (Fig. 3) fundamentally different as observed in *E. histolytica* and *T. vaginalis* (Leitsch et al., 2007, 2009). 2-Nitroimidazole depleted thiol levels in *G. lamblia* most efficiently (Fig. 3), followed by ronidazole and tinidazole, whereas metronidazole had only a modest effect. This is the exact opposite of the situation in *E. histolytica* (Leitsch et al., 2007) and *T. vaginalis* (Leitsch et al., 2009) where metronidazole is more effective than tinidazole and 2-nitroimidazole.

It should be taken into consideration that *G. lamblia* dwells in the small intestine and, therefore, is adapted to rather high concentrations of oxygen but low concentrations of reactive oxygen species due to the presence of bile which contains high concentrations of glutathione (Mastronicola et al., 2011). As compared to *E. histolytica* and *T. vaginalis*, this could be reflected by a different set of factors that influence metabolism of nitroimidazole drugs. Further work will be necessary to identify these factors.

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