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Physiological aspects of nitro drug resistance in *Giardia lamblia*

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ABSTRACT

For over 50 years, metronidazole and other nitro compounds such as nitazoxanide have been used as a therapy of choice against giardiasis and more and more frequently, resistance formation has been observed. Model systems allowing studies on biochemical aspects of resistance formation to nitro drugs are, however, scarce since resistant strains are often unstable in culture. In order to fill this gap, we have generated a stable metronidazole- and nitazoxanide-resistant *Giardia lamblia* WBC6 clone, the strain C4.

Previous studies on strain C4 and the corresponding wild-type strain WBC6 revealed marked differences in the transcriptomes of both strains. Here, we present a physiological comparison between trophozoites of both strains with respect to their ultrastructure, whole cell activities such as oxygen consumption and resazurin reduction assays, key enzyme activities, and several metabolic key parameters such as $NAD(P)^+$/NAD(P)H and ADP/ATP ratios and FAD contents. We show that nitro compound-resistant C4 trophozoites exhibit lower nitroreductase activities, lower oxygen consumption and resazurin reduction rates, lower ornithine-carbamyltransferase activity, reduced FAD and NADP(H) pool sizes and higher ADP/ATP ratios than wildtype trophozoites. The present results suggest that resistance formation against nitro compounds is correlated with metabolic adaptations resulting in a reduction of the activities of FAD-dependent oxidoreductases.

1. Introduction

Giardia lamblia (syn. *G. duodenalis*; *G. intestinalis*), a flagellated, amitochondrial, binucleated protozoan, is the most common causative agent of persistent diarrhea worldwide (Ankarklev et al., 2010; Carranza and Lujan, 2010; Einarsson et al., 2016; Müller and Müller, 2016). Giardiasis is commonly treated with metronidazole (MET), other 5-nitroimidazole compounds (Minenoa and Avery, 2003), nitazoxanide (NTZ) or albendazole (ALB) as an alternative in the case of resistance to nitro drugs (Nash, 2001; Solaymani-Mohammadi et al., 2010; Hemphill et al., 2013). Moreover, *G. lamblia* is susceptible to a variety of antibiotics because of its prokaryote-like transcription and translation machineries (Müller and Hemphill, 2013). According to a commonly accepted model, nitro compounds are activated by reduction yielding toxic intermediates, the electrons being provided by pyruvate oxidoreductase (POR). The reduced nitro compound then binds covalently to DNA and results in DNA breakage and cell death (Brown et al., 1998). Resistance formation to nitro compounds is, however, eagerly detected both *in vitro* and *in vitro.* Studies with metronidazole-resistant strains have revealed, however, that resistance is not always correlated with reduced POR activity thus mechanisms of action independent of POR activity may exist (Upcroft et al., 1990; Upcroft and Upcroft, 1993; Ansell et al., 2015; Leitsch, 2015).

In accordance to the prevailing model for the mode of action of nitro drugs, one would hypothesize that resistant trophozoites have decreased activities of nitroreductases, and that this decrease is due to lower expression levels of the corresponding genes. To verify this hypothesis, freshly obtained, resistant patient isolates would be optimal, but they are difficult to maintain in axenic culture. Therefore, most of the studies compare resistant "model" strains generated *in vitro* with isogenic wildtype strains (Upcroft, 1998). These studies have revealed genome rearrangements (Upcroft et al., 1990, 1992) and profound transcriptional changes evidenced by differential analyses using microarrays followed by quantitative RT-PCR on selected transcripts (Müller et al., 2008) and strand-specific RNA sequencing (Ansell et al., 2017). In both studies, expression profiles of genes coding for variant surface proteins and for genes involved in oxido-reductions – amongst others - are altered the latter allegedly confirming this hypothesis.

These studies on transcriptional changes do not reveal, however, the alterations that occur with respect to the cellular physiology of the resistant lines. Questions such as whether these lines have reduced reductase activities only with nitro drugs or also with other compounds as electron acceptors, and whether they have different pool sizes or ratios of electron and energy providing cofactors, need to be addressed. In this

Abbreviations: ALB, albendazole; MTZ, metronidazole; NTZ, nitazoxanide

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study, we document the physiological changes during resistance formation to nitro drugs in *G. lamblia*, comparing a nitro drug-resistant strain, namely the previously introduced strain C4 (Müller et al., 2007, 2008) and its corresponding wild-type (WBC6) with respect to their ultrastructure, whole cell activities such as oxygen consumption and resazurin reduction assays, functional assays, and pool sizes and ratios of cofactors involved in reductive processes.

2. Materials and methods

2.1. Culture media, biochemicals and drugs

If not otherwise stated, all biochemical reagents were from Sigma (St Louis, MO, USA). Nitazoxanide (NTZ) was synthesized at the Department of Chemistry and Biochemistry, University of Bern, Switzerland (Ch. Leumann). 6-(7-nitro-2,1,3-benzoxadiazol-4-ylthio) hexanol (NBDHEX) was synthesized at the Department of Sciences and Chemical Technologies, University of Rome and kindly provided by M. Lalle (Department of Infectious, Parasitic and Immune-mediated Diseases, Rome, Italy). Albendazole (ALB), NTZ, metronidazole (MET), and NBDHEX were kept as 100 mM stock solutions in DMSO at −20 °C.

2.2. Axenic culture of G. lamblia trophozoites

Trophozoites from *G. lamblia* WB clone C6 wild-type and of the NTZ/MET resistant clone C4 were grown under anaerobic conditions in 10 ml culture tubes (Nunc, Roskilde, Denmark) containing modified TYI-S-33 medium as previously described (Clark and Diamond, 2002). C4 was routinely cultured in the presence of 50 μM NTZ. Subcultures were performed by inoculating 20 μl (wild-type) or 100 μl (C4) of cells from a confluent culture detached by cooling (Müller et al., 2006) to a new tube containing the appropriate medium.

2.3. Harvest and storage of G. lamblia trophozoites

For all experiments comparing wild-type to C4 trophozoites, the medium from confluent cultures was removed one day before the harvest and replaced with fresh medium without NTZ.

Trophozoites were detached by incubation on ice for 15 min followed by centrifugation (300 × g, 10 min, 4 °C). Pellets were washed twice with PBS and either stored at −20 °C (for functional assays) or used directly (determination of dinucleotides and ADP/ATP-ratio, whole-cell assays and RT-PCR).

2.4. Growth curves, determination of minimal inhibitory concentrations, stability of resistance

For all growth studies, *G. lamblia* WBC6 wild-type (WT) and the MET- and NTZ-resistant strain C4 were inoculated into culture tubes (10^4 trophozoites per tube). To determine the respective growth curves, WT and C4 trophozoites were grown with 50 μM NTZ or with equal amounts of DMSO as a solvent control. At various time points, adhering cells were counted in a Neubauer chamber (Müller et al., 2006). To determine minimal inhibitory concentrations (MIC), WT and C4 trophozoites were inoculated in the presence of increasing amounts (dilution series by a factor 2) of the nitro compounds MET, NTZ or NBDHEX, and of ALB as a control. The tubes were incubated at 37 °C for 4 days. The MIC was determined by observing the wells under the microscope starting from higher to lower concentrations. The concentration at which the first living trophozoites were visible is given as the MIC.

2.5. Scanning and transmission electron microscopy

For scanning (SEM) or transmission (TEM) electron microscopy, trophozoites were harvested as described above and processed as described earlier (Müller et al., 2006), with the sole exception that UranyLess EM Stain (Electron Microscopy Sciences, Hatfield, PA) was used instead of uranyl acetate.

Table 1
Primers used in this study. Gene annotations and accession numbers were retrieved from GiardiaDB (giardiadb.org). The genes marked with an asterisk are lateral transfer candidates.

Name	Sequence	GiadiiaDB accession number
ACT_F	ACATATGAGCTGCCAGATGG	Actin related protein
ACT_R	TCGGGGAGGCCTGCAAAC	(GL50803_40817)
FDP_F	TGGGTGGAGCAACAGGGC	A-type flavoprotein;
FDP_R	TTACTGCTTAGGGGCGTTCT	flavodiiron* protein
		(GL50803_10358)
FlaHB_F	GGACAGAGAGGGCGAGGA	Flavohemoprotein*
FlaHB_R	CTAATGGGAGGCCTTGAAG	(GL50803_15009)
NO_F	GCACGACACGCATCATCC	NADH oxidase
NO_R	TTACAGTTTCATCAGCGTGG	(GL50803_9719)
NOLT_F	ACACGGACAGGCCTGGGT	NADH oxidase lateral transfer
NOLT_R	TCAGTCCTTCTTGTTTATCGCAC	candidate* (GL50803_33769)
GlNR1_F	CCTGCTGACAAGGCCGCA	Nitroreductase Fd-NR2*
GlNR1_R	AACACCAATTACTTAAATGTAATG	(GL50803_22677)
GlNR2_F	CTGCAGCTTCACTCAGAGA	Nitroreductase Fd-NR1*
GlNR2_R	TTATTCCACAAACGTTACGTC	(GL50803_6175)
NRfam_F	GGGAATACAAAATGACGGGG	Nitroreductase family protein*
NRfam_R	GTACTCTTCTGTTTGGCGAG	(GL50803_15307)
POR1_F	ATCCAACGCGACCCAGAAG	Pyruvate-flavodoxin-oxido-
POR1_R	GTTCACTGCTTACTCCGCC	reductase (GL50803_17063)
POR2_F	CTCGCACATGGTCCAGGG	Pyruvate-flavodoxin-oxido-
POR2_R	AGAGCCGCAGCCATCTCC	reductase (GL50803_114609)
TrxR_F	CGTTGGCCACGATCCCC	Thioredoxinreductase
TrxR_R	TACTCCTGCATGGCAAGCC	(GL50803_9827)

2.6. RNA analysis and quantification of expression by real-time PCR

For quantification of expression of characterized proteins by real-time PCR after reverse transcription (RT-PCR), trophozoites were grown and harvested as described above. RNA was extracted using the QIAgen RNeasy kit digestion (QIAgen, Hilden, Germany) according to the instructions by the manufacturer. RNA was eluted with RNase-free water and stored at −80 °C. First-strand cDNA was synthesized using the QIAgen OmniscriptRT kit (QIAgen, Hilden, Germany). After quantitative RT-PCR, expression levels were given as relative values in arbitrary units relative to the amount of actin. Quantitative RT-PCR was performed as described (Müller et al., 2008) using the primers listed in Table 1.

2.7. Whole-cell-assays

Oxygen consumption (OCR) and extracellular acidification rates (ECAR) were simultaneously determined (Divakaruni et al., 2014) using a Seahorse XFp device (Agilent, Santa Clara, CA). For each assay, WT or C4 trophozoites were harvested as described and suspended in PBS (2×10^7 cells/ml), and the suspension was added to XFp cell culture miniplates (50 μl per well) containing 150 μl of a sterile NaCl 0.9% (w/v) solution. Plates were centrifuged (400 × g, 2 min, 20 °C) in order to ensure adhesion of the trophozoites. Then, the measurements were performed according to the instructions provided by the manufacturer. During the internal calibration of the XFp extracellular flux cartridge (ca. 20 min), the miniplates containing the trophozoites were incubated at 37 °C and then transferred into the device. OCR and ECAR rates were determined by averaging the rates obtained between 6 and 30 min after the start of the analysis and normalized to the protein contents of the cells.

To determine initial resazurin reduction rates, WT or C4 trophozoites were suspended in PBS or PBS containing 0.2% (w/v) glucose (10^5 trophozoites per ml). 0.1 ml of this suspension were added to 96-well-plates. The assay was started by adding 0.1 ml of resazurin