International Journal for Parasitology: Drugs and Drug Resistance 6 (2016) 148–153

Contents lists available at ScienceDirect

International Journal for Parasitology: Drugs and Drug Resistance

journal homepage: www.elsevier.com/locate/ijppaw

Evaluation of *Giardia lamblia* thioredoxin reductase as drug activating enzyme and as drug target

David Leitsch [a, b, *], Joachim Müller [a], Norbert Müller [a]

[a] Institute of Parasitology, Vetsuisse Faculty, University of Bern, Länggassstrasse 122, CH-3012, Bern, Switzerland
[b] Institute of Specific Prophylaxis and Tropical Medicine, Medical University of Vienna, Kinderspitalgasse 15, A-1090, Vienna, Austria

ARTICLE INFO

Article history:
Received 27 June 2016
Accepted 21 July 2016
Available online 22 July 2016

Keywords:
Giardia lamblia
Thioredoxin reductase
Antigiardial drugs

ABSTRACT

The antioxidative enzyme thioredoxin reductase (TrxR) has been suggested to be a drug target in several pathogens, including the protist parasite *Giardia lamblia*. TrxR is also believed to catalyse the reduction of nitro drugs, *e.g.* metronidazole and furazolidone, a reaction required to render these compounds toxic to *G. lamblia* and other microaerophiles/anaerobes. It was the objective of this study to assess the potential of TrxR as a drug target in *G. lamblia* and to find direct evidence for the role of this enzyme in the activation of metronidazole and other nitro drugs.

TrxR was overexpressed approximately 10-fold in *G. lamblia* WB C6 cells by placing the *trxR* gene behind the arginine deiminase (ADI) promoter on a plasmid. Likewise, a mutant TrxR with a defective disulphide reductase catalytic site was strongly expressed in another *G. lamblia* WB C6 cell line. Susceptibilities to five antigiardial drugs, *i.e.* metronidazole, furazolidone, nitazoxanide, albendazole and auranofin were determined in both transfectant cell lines and compared to wildtype. Further, the impact of all five drugs on TrxR activity *in vivo* was measured.

Overexpression of TrxR rendered *G. lamblia* WB C6 more susceptible to metronidazole and furazolidone but not to nitazoxanide, albendazole, and auranofin. Of all five drugs tested, only auranofin had an appreciably negative effect on TrxR activity *in vivo*, albeit to a much smaller extent than expected. Overexpression of TrxR and mutant TrxR had hardly any impact on growth of *G. lamblia* WB C6, although the enzyme also exerts a strong NADPH oxidase activity which is a source of oxidative stress.

Our results constitute first direct evidence for the notion that TrxR is an activator of metronidazole and furazolidone but rather question that it is a relevant drug target of presently used antigiardial drugs.

1. Introduction

Giardia lamblia (syn *duodenalis*, *intestinalis*) is a microaerophilic protist parasite that occurs in all parts of the world and infects hundreds of millions of people every year (Centers for Disease Control and Prevention, CDC). It colonizes the small intestine and causes gastrointestinal symptoms like nausea, diarrhea, bloating and malabsorption of nutrients. Although not life-threatening in most cases, *Giardia* infections can be persistent and cause growth retardation in children (Buret, 2008). Treatment mainly relies on 5-nitroimidazoles, such as metronidazole and tinidazole, or albendazole, a benzimidazole drug (Leitsch, 2015). 5-nitroimidazoles have been in use against practically all microaerophilic or anaerobic pathogens for more than 50 years due to the comparably low rate of resistance (Leitsch, 2015). However, metronidazole-resistant microaerophiles and anaerobes, including isolates of *G. lamblia*, do occur. Due to the importance of 5-nitroimidazoles, especially metronidazole which is listed among the “essential medicines” by the WHO (World Health Organisation, 2015), a large number of studies on 5-nitroimidazole action and resistance have been conducted throughout the last 30 years.

5-Nitroimidazoles are essentially prodrugs and not very reactive. Reduction at the nitro group, however, activates nitroimidazoles which react with numerous cell constituents - in *G. lamblia e.g.* DNA (Uzlikova and Nohynkova, 2014), proteins (Leitsch et al., 2012), and thiols (Leitsch et al., 2012). Due to the extremely low reduction potential of 5-nitroimidazoles reduction at the nitro group occurs quantitatively only in microaerophilic and anaerobic

* Corresponding author. Institute of Parasitology, Vetsuisse Faculty, University of Bern, Länggassstrasse 122, CH-3012, Bern, Switzerland.
E-mail address: david.leitsch@vetsuisse.unibe.ch (D. Leitsch).

http://dx.doi.org/10.1016/j.ijpddr.2016.07.003

organisms (Müller, 1983). In the protist parasites *G. lamblia*, *Entamoeba histolytica* and *Trichomonas vaginalis*, several enzymatic pathways were identified that are likely to play a role in 5-nitroimidazole reduction, including the central metabolic enzyme pyruvate:ferredoxin oxidoreductase (PFOR) together with ferredoxin (Townson et al., 1996; Rasoloson et al., 2002; Leitsch et al., 2011) and thioredoxin reductase (TrxR) (Leitsch et al., 2007, 2009; 2011), a central redox regulator. Further, another 5-nitroimidazole reducing enzyme, nitroreductase 1 (NR1), was identified in *G. lamblia* (Müller et al., 2007). A correlation between expression levels of nitroreductase 1 and PFOR/ferredoxin and metronidazole sensitivity in *G. lamblia* is well documented, as PFOR and nitroreductase 1 are less strongly expressed in many metronidazole-resistant cell lines (Müller et al., 2008; Leitsch et al., 2011). Moreover, overexpression of NR1 from a plasmid renders *G. lamblia* more sensitive to metronidazole (Nillius et al., 2011). Direct evidence for a role of TrxR in 5-nitroimidazole reduction *in vivo*, however has been missing so far.

Importantly, TrxR was not only found to reduce 5-nitroimidazoles but also to be targeted by reduced nitroimidazole intermediates (Leitsch et al., 2007, 2009; 2011), resulting, at least in *E. histolytica* (Leitsch et al., 2007) and *T. vaginalis* (Leitsch et al., 2009), in a diminished thioredoxin reducing activity of the enzyme (Leitsch et al., 2007, 2009). Thus, TrxR has an intriguing double role as an activator and target of 5-nitroimidazoles. It was hypothesised that inhibition of TrxR could be one of the major toxic effects brought about by 5-nitroimidazoles (Leitsch et al., 2007, 2009). The TrxR/thioredoxin (Trx) redox system has multiple roles in most organisms, including reduction of peroxiredoxins and maintaining the activity of enzymes like ribonucleotide reductase and methionine sulfoxide reductase. In *G. lamblia*, however, the role of the TrxR/Trx system is still poorly understood. Although *G. lamblia* TrxR displays marked disulphide reduction and NADPH oxidase activities (Tejman-Yarden et al., 2013), a functional thioredoxin has not be identified so far (Leitsch et al., 2011; manuscript in preparation). Further, several enzymes known to be dependent on thioredoxin-mediated reduction, such as ribonucleotide reductase, are absent from the parasite. However, despite the current lack of knowledge about the physiological role of TrxR it is generally believed to be an important target not only of metronidazole (Leitsch et al., 2012) but also of auranofin, an antirheumatic drug that has been reprofiled for off-label use against *G. lamblia* (Tejman-Yarden et al., 2013) and *E. histolytica* (Debnath et al., 2012).

In order to evaluate the role of TrxR as an activator of antigiardial drugs and as a drug target, expression levels of the enzyme were strongly increased in *G. lamblia* trophopzoites by transfection of an episomal copy of the TrxR gene under control of the arginine deiminase (ADI) promoter. Likewise, a dominant negative mutant of the TrxR gene under control of the ADI promoter was introduced. Transfectants were assayed for altered drug susceptibilities and used for enzyme inhibition assays.

2. Materials and methods

2.1. Chemicals

Metronidazole, furazolidone, nitazoxanide, auranofin and albendazole were purchased from Sigma (St. Louis, Mo, USA). All drugs tested are depicted in Fig. 1. Growth medium constituents were purchased from Merck (peptone from casein, yeast extract, sodium chloride, glucose, ammonium iron (III) citrate). Fetal calf serum was purchased from Biochrom (Bioswisstec AG, Schaffhausen, Switzerland).

2.2. Cell culture

G. lamblia WB C6 (ATCC 50803) trophozoites were axenically cultivated in Keister's modified Diamond's medium. Media were sterile-filtered. Subcultures were performed every third day.

2.3. Construction of a TrxR overexpressing transfectant

The TrxR gene (GL50803_9827; XM_001707116) was amplified from genomic DNA isolated from WB C6 (ATCC 50803) with primers bearing 50 bp of the upstream region and 50 bp of the downstream region, respectively, of the arginine deiminase gene (GL50803_112103; XM_001705703), and *Pac*I and *Xba*I restriction sites for cloning into the pPac-VInteg vector (Štefanić et al., 2009). The primer sequences were as follows: (forward) CATCTAGAAACGTCTACACGTGAGGTG TGTAAACTTCCGGAGAAAAAAATCCTAGTACATGTCTGCTCAAGCATTCGA, (reverse) CATTAATTAAC TGGATATGAACATGTCAATTATTTGATATCTGAATTACAATTCACTGTTTTAGT GATGGTGATGGTGAT. Transfections of the new plasmid pTrxR into WB C6 trophozoites were performed in a BTX Electro cell manipulator 600 (Harvard Apparatus) with the settings 500 V, 800 μF, and 720 Ω. Transfectant WB C6 cells were selected *via* the plasmid-encoded puromycin N-acetyl-transferase (*pac*) gene by adding puromycin to the growth medium (100 mg/l). The plasmid constructs are schematically depicted in Supplementary Fig. 1.

2.4. Construction of an episomal mutagenized TrxR gene

The second cysteine of the active site of TrxR on pTrxR was mutated to serine using the QuikChange II XL Site-Directed Mutagenesis kit (Agilent) according to the manufacturer's instructions. The mutagenesis primers introduced one single nucleotide exchange in order to alter a cysteine codon (TGC) to a serine codon (AGC). The sequences of the primers were as follows: (forward) GTCGGCCTGCGCTGTCAGCGATTCTGC, (reverse) GCAGAATCGCTGACAGCGCAGGCCGAC. The resulting plasmid pTrxR-mut was transfected into WB C6 as described above.

2.5. Two-dimensional gel electrophoresis of G. lamblia protein extracts (2DE)

Two-dimensional gel electrophoresis (2DE) with *G. lamblia* cell extracts was performed as described previously (Leitsch et al., 2011, 2012). Gels were stained with Coomassie Blue R-250 and evaluated using Melanie™ 4 software (Genebio).

2.6. mRNA quantification of expression by real-time RT–PCR

For quantification of TrxR mRNA expression, cells were harvested as described above and RNA was extracted using a Qiagen RNeasy Kit (Qiagen, Hilden, Germany). Synthesis of first-strand cDNA was performed using a Qiagen OmniscriptRT Kit (Qiagen). The primers used for the amplification of a 189 bp TrxR gene fragment: (forward) CGTTGGCCACGATCCCC, (reverse) GGGGATC GTGGCCAACG. TrxR mRNA levels were calculated using actin mRNA as internal standard (primers: ACTquantF, ACATATGAGCTGCCAGATGG; ACTquantR TCGGGGAGGCCTGCAAAC).

Quantitative RT–PCR was performed on a LightCyclerTM Instrument (Roche Diagnostics, Rotkreuz, Switzerland) as described previously (Nillius et al., 2011). Expression levels of TrxR mRNA were calculated as arbitrary units in relation to the quantity of actin mRNA. PCRs were performed in triplicate in three independent experiments.