Journal of Antimicrobial Chemotherapy (2007) **60**, 280–287
doi:10.1093/jac/dkm205
Advance Access publication 8 June 2007

JAC

Characterization of *Giardia lamblia* WB C6 clones resistant to nitazoxanide and to metronidazole

Joachim Müller*, Maaike Sterk, Andrew Hemphill and Norbert Müller

Institute of Parasitology, University of Berne, Länggass-Strasse 122, CH-3012 Berne, Switzerland

Received 25 January 2007; returned 24 April 2007; revised 26 April 2007; accepted 11 May 2007

***Objectives*: The characterization of *Giardia lamblia* WB C6 strains resistant to metronidazole and to the nitro-thiazole nitazoxanide [2-acetolyloxy-*N*-(5-nitro 2-thiazolyl) benzamide] as the parent compound of thiazolides, a novel class of anti-infective drugs with a broad spectrum of activities against a wide variety of helminths, protozoa and enteric bacteria.**

***Methods*: Issuing from *G. lamblia* WB C6, we have generated two strains exhibiting resistance to nitazoxanide (strain C4) and to metronidazole (strain C5) and determined their susceptibilities to both drugs. Using quantitative RT–PCR, we have analysed the expression of genes that are potentially involved in resistance formation, namely genes encoding pyruvate oxidoreductases (POR1 and POR2), nitroreductase (NR), protein disulphide isomerases (PDI2 and PDI4) and variant surface proteins (VSPs; TSA417). We have cloned and expressed PDI2 and PDI4 in *Escherichia coli*. Using an enzyme assay based on the polymerization of insulin, we have determined the activities of both enzymes in the presence and absence of nitazoxanide.**

***Results*: Whereas C4 was cross-resistant to nitazoxanide and to metronidazole, C5 was resistant only to metronidazole. Transcript levels of the potential targets for nitro-drugs POR1, POR2 and NR were only slightly modified, PDI2 transcript levels were increased in both resistant strains and PDI4 levels in C4. This correlated with the findings that the functional activities of recombinant PDI2 and PDI4 were inhibited by nitazoxanide. Moreover, drastic changes were observed in VSP gene expression.**

***Conclusions*: These results suggest that resistance formation in *Giardia* against nitazoxanide and metronidazole is linked, and possibly mediated by, altered gene expression in drug-resistant strains compared with non-resistant strains of *Giardia*.**

Keywords: antigenic variation, differential gene expression, protein disulphide isomerase, targets

Introduction

Giardia lamblia (syn. *G. duodenalis*; *G. intestinalis*), a flagellated protozoan, is the most common causative agent of persistent diarrhoea worldwide. The life cycle includes motile, flagellated trophozoites parasitizing the upper intestine, and thick-walled cysts forming in the lower intestine that are shed with the faeces.[1] Antigiardial chemotherapy is directed against the trophozoites. In the past, various antiparasitic drugs have been developed but only a few turned out to be effective for treatment of giardiasis.

Since the late 1950s, metronidazole (commercially known as Flagyl®) and other nitroimidazoles have been used as therapy of choice against giardiasis.[2,3] Metronidazole efficiently enters giardial trophozoites and is supposed to be 'activated' via redox mechanisms involving reduction of the nitro group by the metabolic anaerobic enzyme pyruvate:ferredoxin oxidoreductase, PFOR or POR, during the decarboxylation of pyruvate.[4] Reduced metronidazole then binds covalently to DNA, and the resulting damage leads to subsequent death of the parasite. Although metronidazole and other nitroimidazoles are able to efficiently kill *Giardia* trophozoites, treatment of giardiasis with these drugs is often associated with recurrence of symptoms.[5] Furthermore, drug resistance is one of the reasons for treatment failure, but it is difficult to discriminate between 'cure followed by reinfection' and other effects such as lactose intolerance. Resistance to metronidazole and other nitroimidazoles has mostly been demonstrated or induced *in vitro*. However, various genotypically distinct isolates with reduced drug susceptibility have also been found in human patients.[2,6] Formation of giardial

*Corresponding author. Tel: +41-31-6312384; Fax: +41-31-6312477; E-mail: joachim.mueller@ipa.unibe.ch

resistance to metronidazole seems to be associated with down-regulation of POR activity.[3] This is consistent with the involvement of POR in metronidazole activation.[7]

The situation in antigiardial chemotherapy changed in the middle of the 1990s, when a new antiparasitic agent, nitazoxanide (commercially known as Alinia®), was introduced to the market.[8,9] Nitazoxanide is the first agent with proven efficacy in cryptosporidiosis and has recently been approved in the USA for the treatment of cryptosporidiosis and giardiasis in children and adults.[8] In comparison with metronidazole and other nitroimidazoles, nitazoxanide is well tolerated although mild to moderate gastrointestinal disorders such as nausea and diarrhoea have occasionally been associated with higher doses particularly used in immunocompromised persons. However, these side effects are rarely severe and do not require interruption of treatment. Originally developed as a veterinary antihelminthic, nitazoxanide is known to exhibit a broad spectrum of activity against a wide variety of intestinal parasites such as *G. lamblia*, *Entamoeba histolytica*[10] the apicomplexan *Cryptosporidium parvum* and enteric bacteria infecting animals and humans.[11,12]

Nitazoxanide is a nitrothiazolyl-salicylamide, which is well absorbed after oral administration. Upon oral uptake, the drug is rapidly deacetylated to tizoxanide and further metabolized to tizoxanide-glucuronide.[8] Tizoxanide has been reported to display antimicrobial activity similar to nitazoxanide, while tizoxanide-glucuronide was largely inactive against a number of pathogens. Tizoxanide and tizoxanide-glucuronide are further metabolized, or excreted, in urine and/or bile and faeces.

Nitazoxanide represents the parent compound of a class of drugs named thiazolides.[9] Thiazolides lacking the nitro group have been shown to exhibit decreased efficacy against *Giardia*.[10,13] Therefore, an involvement of the nitro group in the mechanism of action, with the participation of POR similar to metronidazole, was postulated.[14] The nitro group is also required for *in vitro* activity against anaerobic bacteria.[15] Inhibition of the anaerobic energy metabolism through nitazoxanide by interfering in functional activity of POR has recently been reported for other protozoan parasites,[16] but this process is thought to be only one of several pathways by which the drug exhibits antiprotozoal activity.[8] In fact, more recent studies have shown that nitazoxanide derivatives lacking the nitro group exhibit profound *in vitro* activity against intracellular pathogens such as *Neospora caninum*, *C. parvum* and *Besnoitia besnoiti*,[9,17] suggesting that the nitro group is not required for the activity against intracellular parasites.

In a previous study, we investigated the *in vitro* effects of nitazoxanide and other thiazolides against *G. lamblia* trophozoites grown either axenically or in co-culture with Caco2 cells[13] and have seen differences in the effects of metronidazole and nitazoxanide on cellular morphology. In order to elucidate the biochemical nature of resistance formation against nitazoxanide and metronidazole, we have now generated metronidazole- and nitazoxanide-resistant clones and compared them with the wild-type WB C6 with respect to their growth behaviour and to the expression pattern of genes that are potentially involved in resistance formation. Besides the two POR genes found in the *Giardia* genome (The *Giardia lamblia* Genome Database; www.mbl.edu/Giardia) and a nitroreductase (NR) that binds to, and is functionally inhibited by nitazoxanide and other thiazolides (J. Müller, S. Sanderson, J. Wastling, N. Müller and A. Hemphill, unpublished results), we have included two protein disulphide isomerases (PDIs) expressed in trophozoites, namely PDI2 and PDI4.[18] PDIs were included in this analysis since in *N. caninum*, PDI has been identified as a tizoxanide binding protein and since recombinant PDI could be inhibited by various thiazolides *in vitro* (J. Müller, A. Naguleswaran, N. Müller and A. Hemphill, unpublished results). Furthermore, since upon selective immunological and/or physiological pressure, *G. lamblia* trophozoite populations may undergo antigenic variation[19,20] and/or initiate encystations,[21] variant surface protein (VSP) and cyst wall protein (CWP) gene expression have also been analysed in this study.

Materials and methods

Tissue culture media, biochemicals and drugs

If not otherwise stated, all biochemical reagents were from Sigma (St Louis, MO, USA). Tizoxanide was synthesized at the Department of Chemistry, University of Liverpool (A. Stachulski), nitazoxanide at the Department of Chemistry and Biochemistry, University of Berne (Ch. Leumann). For structural formulae, see Müller *et al.*[13] They were kept as 100 mM stock solutions in DMSO at 4°C.

Axenic culture of Giardia trophozoites and drug treatment assays

Trophozoites from *G. lamblia* WB clone C6 were grown under anaerobic conditions in 10 mL culture tubes (Nunc, Roskilde, Denmark) containing modified TYI-S-33 medium as described.[13] In order to initiate subcultures, cultures with confluent trophozoite lawns were incubated on ice for 15 min. Suspended living (adherent) trophozoites were counted (Neubauer chamber, 200×). Subcultures were initiated by adding 10^4 trophozoites to a new culture tube.

The effects of drugs were assayed by adding increasing concentrations of nitazoxanide or metronidazole to trophozoite subcultures. Proliferation was monitored by counting living trophozoites after harvest at various time points. For this harvest, medium was removed and replaced with ice-cold PBS in order to count only living (adherent) trophozoites. IC_{50} values were obtained from suitable fittings of the growth curves.[13]

Generation of nitazoxanide- and metronidazole-resistant G. lamblia trophozoites

Nitazoxanide- and metronidazole-resistant *G. lamblia* trophozoites were generated using parasites with the same genetic background (i.e. WB C6). In order to obtain drug-resistant trophozoites,[22] *G. lamblia* WB C6 wild-type parasites grown to confluence were initially cultured in the presence of sublethal concentrations of the two compounds (5 μM nitazoxanide or 10 μM metronidazole, respectively). After 3 days, the medium was removed, viable trophozoites remained attached to the tube walls, and fresh medium was added, containing 7.5 μM nitazoxanide or 15 μM metronidazole, respectively. The status of the trophozoites was monitored microscopically by counting living trophozoites. When the respective numbers remained constant or increased, fresh medium was added, containing similar or slightly higher concentrations of the two drugs. In case the number of living trophozoites decreased, medium was replaced by fresh medium without the corresponding drug.[23] This operation was repeated until levels of 40 μM nitazoxanide or 60 μM metronidazole were reached (Table 1). Clones from resistant

Table 1. Schedule for the establishment of nitazoxanide (NTZ)- and metronidazole (MET)-resistant *G. lamblia* WB C6 lines prior to cloning (at the indicated time points, trophozoite medium was replaced with new medium containing NTZ or MET as indicated)

Time (days)	NTZ (μM)	MET (μM)
0	5	10
3	7.5	15
5	7.5	15
10	10	20
12	10	20
14	10	20
17	15	30
19	0	30
21	10	40
24	15	40
27	20	50
30	20	50
34	25	60
37	25	60
41	transformation into stabilates, storage in liquid nitrogen	
Recovery		
0	0	0
1	20	50
6	25	50
10	25	50
14	40	60

cell lines were generated by two rounds of dilution of trophozoites in drug-free medium and transfer to 96-well plates with an expectance value (λ) of 0.5 trophozoites per well according to the Poisson law $P_\lambda(n) = \lambda^n/n! \times e^{-\lambda}$, n being the number of events and $P_\lambda(n)$ being the probability for n to occur with an expectance value λ. The plates were incubated in an anaerobic incubator (Scholzen Microbiology Systems, Kriems, Switzerland) containing 100% N_2 for 2 days. Wells were then screened for growing trophozoites and the contents of 10 wells with growing trophozoites for each drug resistance were then transferred to normal culture tubes containing drug-free medium and allowed to recover for 1 day. Then, drugs were added (40 μM nitazoxanide and 60 μM metronidazole, respectively). The clones having maintained their resistance (four in the case of nitazoxanide and six in the case of metronidazole) were subcultivated. The clones with the best growth, i.e. the nitazoxanide-resistant clone C4 and the metronidazole-resistant clone C5, were selected for detailed characterization.

Processing of RNA samples and quantitative RT–PCR

To quantify gene expression by real-time RT–PCR, trophozoites of WB C6 wild-type, clones C4 and C5 were grown in the absence of drugs until near confluence was reached. Cells were harvested as described and RNA was extracted using the Qiagen RNeasy™ kit and including a DNase I digestion (to remove residual genomic DNA) according to the manufacturer's instructions. RNA was eluted with 50 μL RNase-free water and stored at −80°C.

First strand cDNA was synthesized using the Qiagen Omniscript™ RT kit as described by the manufacturer with random primers for subsequent ACT-, CWP1-, GDH-, NR-, PDI2-, PDI4,- POR1-, POR4- and 16S rRNA-PCR or with a polyT-ANC primer[24] for subsequent VSP^{tot}-, TSA417- and ACT-PCR (for primer sequences, see Table 2). Overall amplifications of cDNA molecules representing analogues of mRNA from different VSP genes (VSP^{tot}) were performed by 3′ RACE PCR as previously described.[24] Briefly, amplification reaction mixes included a forward primer (primer VSP^{tot}) complementary to a highly conserved VSP gene region (MM16 region, as described previously;[25] see Table 2) and a reverse anchor (ANC) primer (Table 2).[24] Quantitative PCR was done with 4 μL of 1:100-diluted cDNA using the Quanti Tect™ SYBR Green PCR Kit (Qiagen) in a 10 μL standard reaction containing a 0.5 μM concentration of forward and reverse primers (MWG Biotech, Ebersberg, Germany). Furthermore, a control PCR included RNA equivalents from samples that had not been reverse transcribed into cDNA (data not shown) to confirm that no DNA was amplified from any residual genomic DNA that might have resisted DNase I digestion (see above). PCR was started by initiating the 'Hot-Start' *Taq* DNA polymerase reaction at 95°C (15 min). Subsequent DNA amplification was done in 40 cycles including denaturation (94°C, 15 s), annealing (60°C, 30 s) and extension (72°C, 30 s); temperature transition rates in all cycle steps were 20°C/s. Fluorescence was measured at 82°C with primers for ACT-, CWP1-, GDH-, NR-, PDI2-, PDI4,- POR1-, POR2- and 16S rRNA-PCR or 79°C for VSPtot-and TSA417-PCR during the temperature shift after each annealing phase. From the quantitative RT–PCR, mean values (±SE) from triplicate determinations were assessed and expression levels of the genes summarized in Table 2 were given as values in arbitrary units relative to the amount of 16S rRNA (for mRNA representing ACT, CWP1, GDH, NR, PDI2, PDI4, POR1 and POR2) or the constitutively expressed 'house keeping' gene ACT (for mRNA representing VSP^{tot} and TSA417).

Cloning and heterologous expression of G. lamblia PDI2 and PDI4

In order to clone GlPDI2 and GlPDI4 into the His-tag-expression vector pET151 directional TOPO (Invitrogen, Carlsbad, Canada), primers were created for the amplification of gene fragments encoding the PDI polypeptides without signal peptides (Table 2). CACC at the 5′ end was added in order to allow directional cloning (MWG Biotech, Ebersberg, Germany).

For amplification by PCR, DNA was extracted from 10^6 trophozoites using the DNeasy™ kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and eluted from the spin column with 50 μL ultrapure water. The PCR was performed using 0.6 U of *Pfu* (Promega, Madison, WI, USA), 2 μL of *Pfu* buffer 10× (Promega), 20 pmol of each primer, 0.16 mM dNTPs (Promega) and 0.1–1 μL of DNA diluted 1/10 in a total volume of 20 μL. The amplification was performed at 56°C (PDI2) or 54°C (PDI4) annealing temperature during 28 cycles in a GeneAmp™ PCR System 9700 thermocycler (Applied Biosystems, Foster City, CA, USA). The resulting products were inserted into pET151 using the respective cloning kit according to the manufacturer's instructions. The vector was transformed into *Escherichia coli* TOP 10 cells (Invitrogen).

For heterologous expression of recombinant PDIs, miniprep DNA from *E. coli* TOP 10 containing the plasmid with the insert in correct orientation was batch transformed into *E. coli* BL21 Star (Invitrogen). The batch was grown overnight in 2 mL of LB with 100 ppm carbenicillin, then transferred to a 500 mL Erlenmeyer containing 50 mL of LB with 100 ppm carbenicillin and grown until A_{600} 0.5 was reached. Expression of recombinant PDIs was induced by addition of 1 mM IPTG. As a negative control, 10 mL of culture

Table 2. Overview of primers used in this study

Gene Accession number	Region of CDS or rRNA	Primer (5′ → 3′)
Actin (ACT) EAA39190	CDS 715–933	ACTquantF ACATATGAGCTGCCAGATGG ACTquantR TCGGGGAGGCCTGCAAAC
Cyst wall protein 1 (CWP1) XM 766142.1	CDS 367–713	CWP1quantF GGCGATATTCCCGAGTGCATGTG CWP1quantR GTGAGGCAGTACTCTCCGCAGT
Glutamate dehydrogenase (GDH) XM_773614	CDS 761–893	GDHquantF AGGTCCTCACCTTCTCAGACT GDHquantR GGATACTTGTCCTTGAACTCGG
Nitroreductase (NR) EAA43030.1	CDS 526–794 plus 8 of 3′ utr.	NRquantF CCTGCTGACAAGGCCGCA NRquantR AACACCAATTACTTAAATGTAATG
Protein disulphide isomerase 2 (PDI2) EAA42483.1	CDS 1055–1350 plus 13 of 3′ utr. CDS 57–1350 plus 12 of 3′ utr.	PDI2quantF GGCCCAGGGCGAGGAGT PDI2quantR AGACAAGAACCGTTTACTTCTT PDI2fullF CACCTTGGTTCTCACGCAAGACA PDI2fullR GACAAGAACCGTTTACTTCT
Protein disulphide isomerase 4 (PDI4) AF295634.1	CDS 46–1065 CDS 775–1065 plus 1 of 3′ utr.	PDI4fullF CACCGAGGTGCTCGTTCTCCA PDI4fullR TCAGAGCTCCTTGTCCCC PDI4quantF CCGAAGGACGAGACTTCCT PDI4quantR CTCAGAGCTCCTTGTCCCC
Pyruvate oxidoreductase 1 (POR1) *Giardia* DB orf:17063	CDS 3341–3600 plus 9 of 3′ utr.	POR1quantF ATCCAACGCGACCCAGAAG POR1quantR GTTCACTGCTTACTCCGCC
Pyruvate oxidoreductase 2 (POR2) *Giardia* DB orf:114609	CDS 3325–3547	POR2quantF CTCGCACATGGTCCAGGG POR2quantR AGAGCCGCAGCCATCTCC
Ribosomal RNA 16S (16S rRNA) M54878.1	CDS 79–300	Gl16SquantF GACGGCTCAGGACAACGG Gl16SquantR CTCTCCGGAGTCGAACCC
Variant surface protein TSA417 U89152.1	CDS 127–335	TSA417quantF TGTGGAACGTGTGCCAATAG TSA417quantR AGACACGTAGTACAGTCGG
Variant surface protein (total)	see Von Allmen *et al.*[24]	VSP_{tot}quantF (MM16) GGCTTCCTCTGCTGGTGGTTC VSP_{tot}quantR (ANC) GACCACGCGTATCGATGTCGA

CDS, coding sequence; utr., untranslated.

was withdrawn prior to induction and cultivated in a 100 mL Erlenmeyer. Cells were harvested after 3.5 h, chilled down, pelleted by centrifugation (4000 **g**, 20 min, 4°C) and stored at −20°C.

For His-tag purification, pellets from induced *E. coli* cells were suspended in 0.5 mL of 1× LEW buffer (Macherey-Nagel, Düren, Germany) containing 0.5% Triton X-100 and 10 μL of protease inhibitor cocktail suitable for His-tag purification (Sigma P8849). The suspension was freeze-thawed three times on dry ice, then centrifuged for 15 min at 13 000 **g** and 4°C. The extraction was repeated once. Supernatants were combined and loaded on Protino Ni-TED 150 columns (Macherey-Nagel) in tandem. Columns were washed with 1× LEW buffer, proteins were eluted with elution buffer according to the manufacturer's instructions. Eluted protein was stored in 50% glycerol at −20°C.

PDI enzyme assay

PDI activity was measured by a turbidimetric assay based on the polymerization of reduced insulin.[18,26,27] The assay was performed in 96-well microtitre plates (Nunc). Per well, 80 μL of assay buffer (potassium phosphate 100 mM, pH 7.0) was mixed with 10 μL of bovine pancreas insulin (1.6 mM, dissolved in 0.1 M HCl) and enzymes (0–5 μL) or buffer (substrate blank). The reaction was started by addition of 5 μL of dithiothreitol (20 mM in assay buffer). After 2 min of preincubation, absorbance at 630 nm was read at various time points (0–20 min) on a 96-well plate reader (MRXII, Dynex, Chantilly, VA, USA). Enzyme activity was calculated from the linear increase in turbidity over time.

Results

Generation of nitazoxanide- and metronidazole-resistant G. lamblia trophozoites

By axenic culture of *G. lamblia* WB C6 trophozoites in the presence of slowly and continuously increasing concentrations of nitazoxanide and metronidazole, respectively (Table 1), two drug-resistant trophozoite lines were generated, transformed into stabilates, stored in liquid nitrogen and subsequently recovered in trophozoite medium. These trophozoite lines were subcultured in the presence of up to 40 μM nitazoxanide and 60 μM metronidazole, respectively. Clones from resistant cell lines were generated and growth of the nitazoxanide-resistant clone C4 and the metronidazole-resistant clone C5 was tested on normal medium. All three clones reached comparable densities at 4 days after inoculation, C5 started however, with a delay (Figure 1). Upon prolonged growth in drug-free medium for more than 10 generations, resistance was maintained. The clones C4 and C5 encysted as well as the wild-type when put on encystation medium (data not shown).

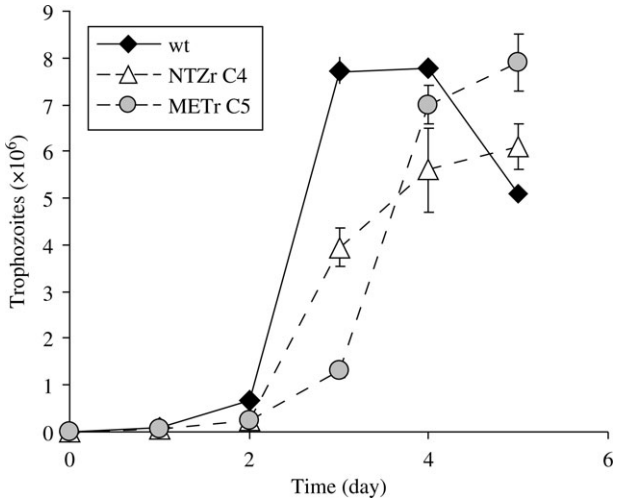

Figure 1. Growth of *G. lamblia* WB C6 wild-type (wt), nitazoxanide-resistant (NTZr C4) and metronidazole-resistant (METr C5) trophozoites. At day 0, 10^4 trophozoites were inoculated to normal culture medium. Trophozoite numbers per culture tube are given as mean values (±SE) from quadruplicate determinations.

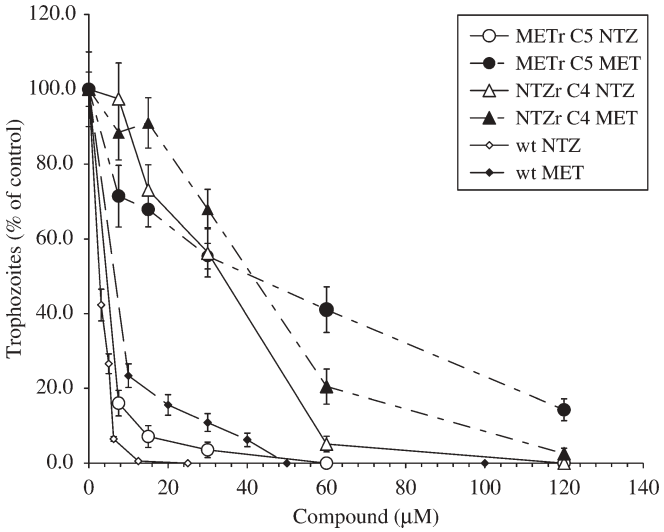

Figure 2. Trophozoite numbers in the presence of metronidazole (MET) or nitazoxanide (NTZ). At day 0, 10^4 trophozoites of *G. lamblia* WBC6 wild-type (wt), nitazoxanide-resistant (NTZr C4) and metronidazole-resistant (METr C5) were inoculated to culture medium containing increasing amounts of nitazoxanide (white symbols) or metronidazole (black symbols). Trophozoites were harvested at day 4 after inoculation. Trophozoite numbers are given as mean values (±SE) from quadruplicate determinations.

Cross-resistance

In order to determine IC_{50} values of the resistant clones C4 and C5 in the presence of nitazoxanide and metronidazole, trophozoites of the respective strains were grown for 4 days at various drug concentrations. The nitazoxanide-resistant clone C4 exhibited cross-resistance to metronidazole with IC_{50} values of 37 μM for nitazoxanide and 59 μM for metronidazole, whereas the metronidazole-resistant clone C5 was resistant to metronidazole only (IC_{50} 42.5 μM) while no resistance to nitazoxanide was noted (IC_{50} 3.6 μM) (Figure 2). The IC_{50} values for the wild-type WB C6 were 1.8 μM for nitazoxanide and 8.1 μM for metronidazole as estimated after logarithmic transformation of the curves shown in Figure 2.

In order to investigate whether resistance formation in the case of the clone C4 was a multidrug resistance phenomenon, C4 and wild-type cells were grown in the presence of non-nitro drugs, namely albendazole and the isoflavonoids genistein, daidzein and formononetin, all described as powerful antigiardial agents.[28,29] Both wild-type and C4 trophozoites were equally sensitive to these compounds (growth curves not shown).

Gene expression

In order to quantify expression potentially involved in resistance formation, we developed a quantitative real-time RT–PCR assay using reverse transcripts of 16S rRNA as reference for the amount of total cDNA. The gene expression level of actin (ACT) was not affected in the clones C4 and C5 as compared with the wild-type. Interestingly, gene expression levels of glutamate dehydrogenase (GDH) were decreased to 37% of the wild-type levels in clone C4 and to 59% in clone C5. As a marker for encystation-related genes, we investigated 'background' CWP1 gene expression in trophozoites. Surprisingly, expression was decreased to 44% of wild-type levels in both strains (Figure 3).

POR is encoded by two genes in the *Giardia* genome, namely POR1 and POR2. In wild-type trophozoites, POR2 had 7.2-fold higher gene expression levels than POR1. In the clone C5, expression of both genes was slightly decreased to 68 (POR1) and 66% (POR2) of their respective wild-type levels (Figure 3).

Expression of a *Giardia* NR was assessed in wild-type versus resistant parasites. This enzyme had expression levels one

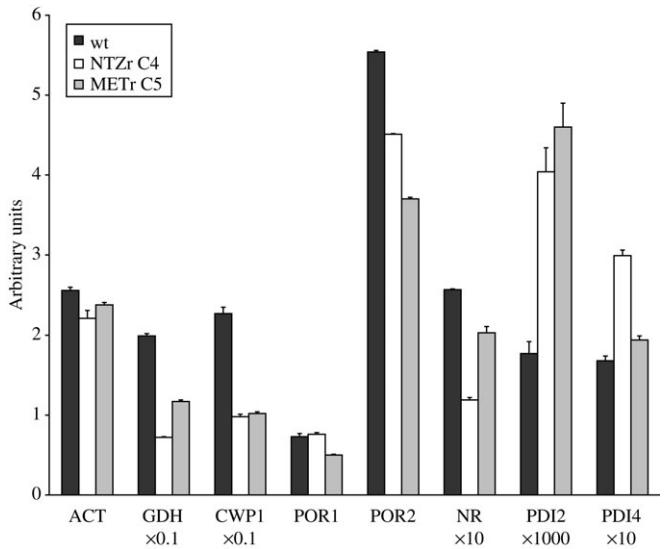

Figure 3. Quantification of gene expression by RT–PCR. Trophozoites were grown in normal culture medium until near confluency was reached. RNA was extracted and reverse transcribed to cDNA. Transcripts of actin (ACT), glutamate dehydrogenase (GDH) and pyruvate oxidoreductase (POR1 and 2), nitroreductase (NR), protein disulphide isomerase (PDI2 and 4) and cyst wall protein (CWP1) were quantified in relation to 16S rRNA. Amounts are expressed in arbitrary units (AU). Mean values (±SE) for triplicates are given. Note that for reasons of scale, GDH and CWP1 values are multiplied by 0.1, NR and PDI4 values by 10 and PDI2 values by 1000. wt, wild-type.

magnitude lower than the POR genes and the ACT gene. In the clones C4 and C5, NR expression levels were only slightly decreased (69% of wild-type level in C4 and 78% of wild-type level in C5; Figure 3).

In addition, we analysed expression levels of PDI2 and PDI4, two major PDIs in *Giardia*. The expression level of PDI2 was approximately three magnitudes lower than the actin expression level. PDI2 gene expression was significantly increased in both resistant clones (230% of wild-type level in clone C4 and 255% in clone C5) PDI4 exhibited two magnitudes higher gene expression levels as compared with PDI2. In the nitazoxanide-resistant clone C4, PDI4 gene expression was increased to nearly twice the wild-type levels, whereas in the metronidazole-resistant clone C5, PDI4 gene expression levels remained nearly unchanged (Figure 3).

Recombinant PDI is inhibited by nitazoxanide

When subjected to a PDI enzyme assay based on the polymerization of reduced insulin, bacterially expressed and purified recPDI4 was shown to be functionally active and catalysed the cross-linking of insulin monomers. Classical PDI inhibitors like bacitracin[30] and *para*-chloro-mercuri benzoic acid (PCMBA) readily inhibited the activity of the recPDI4. Interestingly, also nitazoxanide and tizoxanide, but not metronidazole, inhibited recPDI4 activity (Figure 4). Similar results were obtained with using recPDI2 where 50 μM nitazoxanide and tizoxanide showed inhibition to $<20\%$ of the control levels (data not shown).

VSP gene expression is strongly affected in nitazoxanide- and metronidazole-resistant G. lamblia clones

The most dramatic differences between wild-type and resistant *G. lamblia* clones were found when expression of VSP genes was investigated. For respective analyses, cDNA was synthesized issuing from polyT-ANC primers instead of random oligo primers (see the Materials and methods section). This experimental strategy allowed RT–PCRs to be performed with a general forward primer (MM16 primer) targeted to a highly conserved VSP gene region and a reverse primer targeted to the 3′ terminal ANC sequence adjacent to the polyT stretch of the cDNA molecules (Figure 5). By comparative quantitative RT–PCR, the cDNA synthesized with polyT-ANC was also amplified by including a primer pair specific for the gene TSA417 that encodes the major VSP in WB C6 (Figure 5). Values from both PCRs were determined relative to the ACT gene expression, which was shown to be constant in wild-type and resistant strains (Figure 3). While wild-type clone WB C6 exhibited TSA417 gene expression, corresponding expression levels were dramatically reduced and virtually non-detectable in resistant clones C4 and C5. Conversely, the overall expression of VSP genes (VSP^{tot}) remained constant, suggesting that resistance was associated with an antigenic switch from TSA417 to one, or several unknown VSPs (Figure 5).

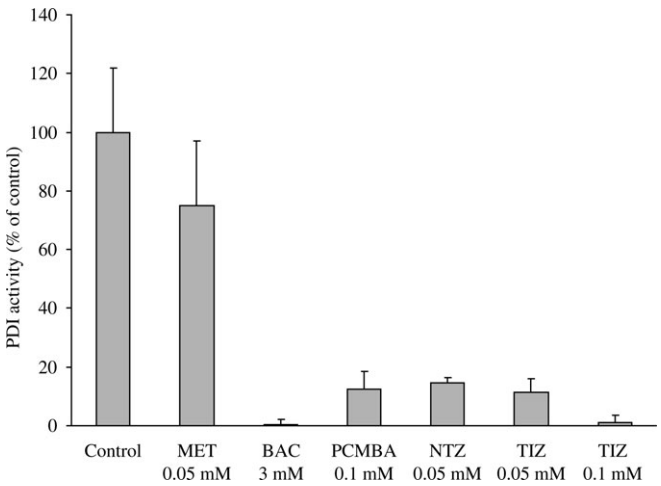

Figure 4. PDI activity assay based on the reduction and polymerization of insulin with recombinant PDI4. Inhibition test with known PDI inhibitors, namely bacitracin (BAC; 3 mM) and *para*-chloro-mercuri benzoic acid (PCMBA; 0.1 mM), metronidazole (MET; 50 μM), nitazoxanide (NTZ; 50 μM) and tizoxanide (TIZ; 50 and 100 μM). Assays were run in triplicate. Bars correspond to mean values (±SE).

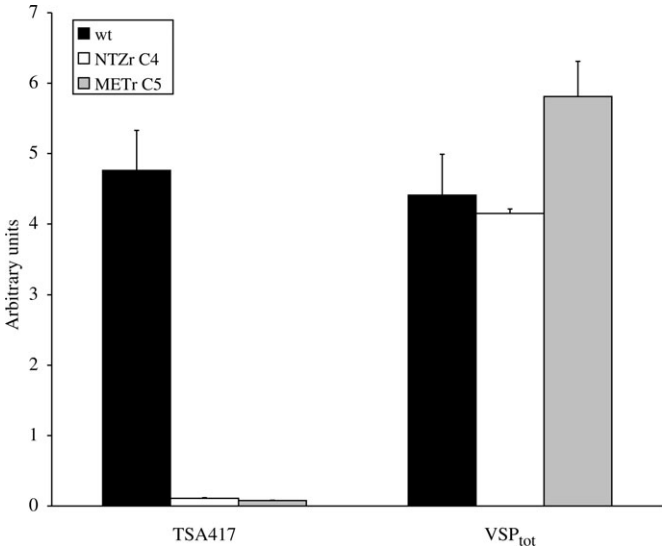

Figure 5. Quantification of variant surface protein (VSP) gene expression by real-time RT–PCR. Trophozoites were grown in normal culture medium until near confluence was reached. RNA was extracted and reverse transcribed to cDNA. Transcripts of the VSP gene encoding TSA417 and total VSPs (VSP^{tot}) were quantified in relation to actin cDNA. Amounts are expressed in arbitrary units (AU). Mean values (±SE) for triplicate determinations are given. wt, wild-type; NTZr, nitazoxanide-resistant; METr, metronidazole-resistant.

Discussion

Our present investigations have demonstrated that giardial resistance to nitazoxanide can be triggered *in vitro*, and that nitazoxanide resistance formation resulted in a simultaneous increase of resistance to metronidazole. Conversely, *in vitro* generated metronidazole resistance turned out to mediate no 'cross-resistance' to nitazoxanide. In particular, we have been successful in generating *in vitro* the nitazoxanide-resistant clone C4 and the metronidazole-resistant clone C5 of *G. lamblia* WB C6. The two clones do not exhibit the same resistance pattern since C4 is cross-resistant to metronidazole, whereas C5 is sensitive to nitazoxanide. This latter finding is in agreement with observations in *Helicobacter pylori* where nitazoxanide-resistant strains did

not exhibit cross-resistance to metronidazole.[23] To our knowledge, clinically relevant resistance to nitazoxanide has never been reported to date, either in *H. pylori*,[31] *Giardia* or in *Cryptosporidium*,[8,12] while metronidazole-resistant isolates of *H. pylori* and *G. lamblia* have been obtained from infected patients.[31]

Although nitazoxanide and metronidazole exhibit structural similarities, the two compounds differentially affect *Giardia* in terms of morphological alterations.[13] In addition, the differences in respective resistance patterns as outlined above suggest differences in their mode of action. Earlier findings had demonstrated that reduction of the nitro group is crucial for metronidazole activation,[7] since oxidoreductases such as POR or other NRs that are present in anaerobic or microaerophilic microorganisms will convert the nitro group into a toxic radical. In addition, in these organisms, resistance to metronidazole was associated with the functional inhibition of POR activity.[3,4,32,33]

While it has been recently shown that nitazoxanide and tizoxanide both inhibit the POR activities in *Giardia*, *Trichomonas*, *Entamoeba* and several anaerobic bacteria,[16] there is no evidence that the thiazole-associated nitro group is actually reduced upon treatment of these organisms. In fact, this study suggests that unreduced nitazoxanide inhibits POR activity by interfering with its cofactor thiamine pyrophosphate.[16] In any case, the presence of the nitro group was also found to be crucial for the antigiardial activity of several nitro-thiazolides with low inhibitory constants.[13]

Taking these findings into account, we decided to perform an RT–PCR-based investigation to assess the POR gene expression levels in the resistant clones C4 and C5. Here, the nitazoxanide-resistant clone C4 did not exhibit significant reduction of expression of the two POR genes, POR1 and POR2, but clearly expression levels were reduced in the metronidazole-resistant clone C5 (Figure 3). This indicates that metronidazole resistance formation may occur in at least two different manners including either reduction of POR gene expression or an as yet unknown pathway that does not depend on an alteration of the respective enzymatic activity.

Additional investigations on the expression of NR, another enzyme potentially involved in nitro reduction (Figure 3), show that both giardial nitazoxanide and metronidazole resistance formation do not lead to significant alterations in NR gene expression. NR has been recently isolated from *Giardia* by affinity chromatography using tizoxanide-epoxy-agarose affinity chromatography, it was shown to bind to nitazoxanide and tizoxanide, and these two compounds and series of other nitro-thiazolides were shown to inhibit the functional activity of recombinant NR *in vitro* (J. Müller, S. Sanderson, J. Wastling, N. Müller and A. Hemphill, unpublished results). Conversely, transcript levels of the highly expressed GDH gene were significantly lowered in both resistant strains. On the metabolic level, this down-regulation of GDH could lead to a reduction of the electron flow to NAD(P)H and thus ultimately to potential reducers of the nitro group from nitazoxanide and/or metronidazole, respectively. If the GDH-dependent redox pathway was in fact involved in activation of the two nitro compounds, down-regulation of GDH or related genes would be an obvious mechanism to mediate nitazoxanide and/or metronidazole resistance formation in *Giardia*.

Further analysis of gene expression levels of PDI2 and PDI4 resulted in the intriguing findings that PDI2 expression levels were increased in both clones, C4 and C5, and PDI4 transcript levels were higher in clone C4 (Figure 3). Further, we found that nitazoxanide and tizoxanide both inhibited the functional activities of PDI2 and PDI4 (see Figure 4). These observations demonstrated that nitazoxanide interferes with PDI functions, and also suggested that up-regulation of PDI expression during resistance formation may compensate for nitazoxanide-mediated loss of PDI activity. PDIs have to be considered as potential targets for antigiardial drug treatment since they are ubiquitous and important players in the maintenance of the cellular redox status by refolding proteins through the rearrangement of disulphide bridges.[34–36] While, as described above, the nitazoxanide- and metronidazole-resistant strains differ from their isogenic wild-type WB C6 with respect to the expression patterns of genes encoding metabolic enzymes (GDH, POR2, PDI1 and PDI2), it is important to note that the by far most drastic regulatory effect was observed for the TSA417 gene locus, encoding the major surface antigen (VSP C6) of WB C6. Expression of TSA417 was massively down-regulated in both resistant strains. As shown in our previous work, *G. lamblia* is able to escape host defence mechanisms by changing the expression of its surface antigens.[19,20] The phenomenon of antigenic variation indicates that *G. lamblia* has adopted the capability to alter its gene expression profile in response to an environmental pressure. As reported recently, epigenetic mechanisms probably including gene acetylation seem to be responsible for antigenic variation.[37] Similar adaptive gene-regulatory processes may cause pleiotropic effects that mediate, or at least participate in, antigiardial drug resistance formation. This adaptive mechanism may include up-, or down-regulation, of the expression of proteins involved in drug interaction, or of genes having direct, or indirect, effects on components involved in the mode of action of the drugs. In future, we will apply various genomic and proteomic approaches to assess the complexity of those cellular functions that are possibly involved in (multi-) drug resistance formation of the parasite.

Acknowledgements

We are indebted to Christian Leumann (Department of Biochemistry and Chemistry, University of Berne) for the synthesis of the thiazolides. This study was supported by grants from the Swiss Secretariat for Education and Science (SBF, COST Action B22; grant no. SBF C05.0104; N. M. and J. M.) and the Swiss National Science Foundation (grant no. 3100A0-112532/1; A. H.). J. M. was partially financially supported by the University of Berne.

Transparency declarations

None to declare.

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