

## Nitroreductase (GINR1) increases susceptibility of *Giardia lamblia* and *Escherichia coli* to nitro drugs

Dorothea Nillius, Joachim Müller and Norbert Müller\*

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

\*Corresponding author. Tel: +41-31-6312474; Fax: +41-31-6312477; E-mail: [norbert.mueller@ipa.unibe.ch](mailto:norbert.mueller@ipa.unibe.ch)

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**Objectives:** The protozoan parasite *Giardia lamblia* causes the intestinal disease giardiasis, which may lead to acute and chronic diarrhoea in humans and various animal species. For treatment of this disease, several drugs such as the benzimidazole albendazole, the nitroimidazole metronidazole and the nitrothiazolidine nitazoxanide are currently in use. Previously, a *G. lamblia* nitroreductase 1 (GINR1) was identified as a nitazoxanide-binding protein. The aim of the present project was to elucidate the role of this enzyme in the mode of action of the nitro drugs nitazoxanide and metronidazole.

**Methods:** Recombinant GINR1 was overexpressed in both *G. lamblia* and *Escherichia coli* (strain BL21). The susceptibility of the transfected bacterial and giardial cell lines to nitazoxanide and metronidazole was analysed.

**Results:** *G. lamblia* trophozoites overexpressing GINR1 had a higher susceptibility to both nitro drugs. *E. coli* were fully resistant to nitazoxanide under both aerobic and semi-aerobic growth conditions. When grown semi-aerobically, bacteria overexpressing GINR1 became susceptible to nitazoxanide.

**Conclusions:** These findings suggest that GINR1 activates nitro drugs via reduction yielding a cytotoxic product.

**Keywords:** drug susceptibility, stable expression, transfection

### Introduction

*Giardia lamblia* (syn. *Giardia duodenalis*; *Giardia intestinalis*), a flagellated protozoan, is the most common causative agent of persistent diarrhoea worldwide.<sup>1</sup> For anti-giardial chemotherapy only a few effective drugs are available, namely the nitroheterocyclic drugs tinidazole, metronidazole, furazolidone, the substituted acridine quinacrine, the aminoglycoside paromomycin and the benzimidazole albendazole.<sup>2–4</sup> In 2000, the nitrothiazolidine nitazoxanide was introduced as an alternative option.<sup>5,6</sup>

Concerning the mode of action of metronidazole and related drugs with nitro groups, the current evidence suggests that the nitro group is reduced to a toxic nitro radical via a redox mechanism involving the pyruvate:ferredoxin oxidoreductase (PFOR) system<sup>3,7</sup> or other enzymes such as nitroreductases.<sup>8</sup> However, it is not clear how nitazoxanide exerts its antiparasitic activity. Nitazoxanide inhibits recombinant bacterial PFORs by a mechanism involving the abstraction of a proton from the thiamine pyrophosphate vitamin co-factor of PFOR through the nitazoxanide anion, thereby inhibiting the production of acetyl-coenzyme A and CO<sub>2</sub> necessary for energy metabolism.<sup>9,10</sup> According to this model, nitazoxanide would display its antimicrobial activity without the involvement of nitro reduction.<sup>10</sup>

We have previously identified a nitroreductase from *G. lamblia* (GINR1) as a nitazoxanide-binding protein. The activity of recombinant GINR1 on dinitrotoluene was inhibited *in vitro* by nitazoxanide. However, reduction of nitazoxanide or metronidazole *in vitro* could not be detected.<sup>11</sup> In another study, we found that a nitazoxanide-resistant clone of *G. lamblia* WBC6 exhibited significantly lower GINR1 mRNA levels compared with the wild-type WBC6.<sup>12</sup>

These findings suggested the following three hypotheses for the involvement of GINR1 in the mode of action of nitazoxanide: (i) GINR1 activates nitazoxanide by partial reduction, yielding a toxic radical or a nitroso or hydroxylamine intermediate that could be even more relevant for toxicity than the radical;<sup>13</sup> (ii) GINR1 inactivates nitazoxanide by full reduction, yielding the corresponding amine, which is inactive;<sup>14</sup> (iii) independently of the reduction of xenobiotics, GINR1 has an essential function in an unknown metabolic pathway that is inhibited via the binding of nitro drugs.

In order to distinguish between these hypotheses, we have chosen a reverse genetics approach, by overexpressing GINR1 in *G. lamblia* and *Escherichia coli* and determined the susceptibilities of the resulting lines to nitazoxanide and metronidazole as compared with control, glucuronidase-transformed recombinant

lines. Furthermore, we have analysed the expression of GINR1 in nitazoxanide-resistant lines of *G. lamblia*.

## Materials and methods

### Tissue culture media, biochemicals and drugs

If not otherwise stated, all biochemical reagents were from Sigma (St Louis, MO, USA). Nitazoxanide was synthesized at the Department of Chemistry and Biochemistry, University of Berne (C. Leumann). Nitazoxanide, metronidazole and albendazole were kept as 100 mM stock solutions in DMSO at  $-20^{\circ}\text{C}$ .

### Axenic culture of *Giardia trophozoites*

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.<sup>15</sup> The nitazoxanide-resistant clone C4<sup>12</sup> was grown in the presence of  $40\ \mu\text{M}$  nitazoxanide. Twenty-four hours prior to an experiment, C4 trophozoites were transferred to drug-free, modified TYI-S-33 medium. Trophozoites were detached by incubation on ice for 30 min. Suspended motile trophozoites were counted (Neubauer chamber). Subcultures were initiated by adding 2–20  $\mu\text{L}$  of trophozoites in a new culture tube. Trophozoites were grown to near confluence and harvested by centrifugation (600 g, 15 min,  $4^{\circ}\text{C}$ ). Trophozoite pellets were washed three times with PBS, pH 7.2, and stored at  $-80^{\circ}\text{C}$ .

### Overexpression of recombinant GINR1

Cloning and heterologous expression of GINR1 and  $\beta$ -glucuronidase A (GusA) in the *E. coli* His-tag expression vector system pET151 (pET151 directional TOPO; Invitrogen, Carlsbad, CA, USA) were carried out as previously described.<sup>11,16</sup> Clones with high expression levels of the recombinant proteins<sup>11,16</sup> were used for the re-cloning of the GINR1 and the GusA open reading frames into the vector pPacV-Integ (kindly provided by A. Hehl, Institute of Parasitology, Zürich, Switzerland). Applying the XbaI and PacI sites of the vector for integration,<sup>17</sup> a forward primer was designed starting with the XbaI site followed by the constitutive glutamate dehydrogenase (GDH) promoter.<sup>18</sup> In the reverse primer, a sequence encoding a human influenza haemagglutinin (HA) tag was included 5' of the PacI site (Table 1). PCRs were performed using *Pfu* polymerase (Promega, Madison, WI, USA), and fragments were cloned into the Zero Blunt TOPO vector (Invitrogen) according to the

manufacturer's instructions. Inserts were excised with XbaI and PacI and ligated into pPacV thus yielding pPacV-GINR1 or pPacV-GusA. Prior to transfection, the vectors were linearized by digestion with SmaI in order to allow chromosomal integration of the transgene by homologous recombination. Then, transfection of *G. lamblia* WBC6 and selection of stable transfectants containing GusA or GINR1 integrated into the genome was performed as previously described via resistance to the antibiotic puromycin.<sup>17</sup>

### Antibodies and western blotting

Crude rabbit anti-GINR1 serum was contracted from GenicBio BioTech (Hong Kong, China). The animal had been immunized with a cocktail of two keyhole limpet haemocyanin (KLH)-coupled peptides representing GINR1 coding sequence (CDS) 10–22 (RKYHPEPLPKEDL-C) and CDS 58–70 (IAKEVSKNPRYSK-C). Antibodies were affinity-purified on recombinant GINR1<sup>11</sup> as described previously.<sup>19</sup> Mouse monoclonal antibodies directed against  $\alpha$ -tubulin of *Trypanosoma brucei* and exhibiting cross-reactivity to the analogous protein of *G. lamblia* clone WBC6 was kindly provided by K. Gull (Dunn School of Pathology, University of Oxford, UK).

Total protein from *E. coli* and *G. lamblia* lines was separated by SDS-PAGE (10% gels) under reducing conditions.<sup>20</sup> Western blots were prepared by electrophoretically transferring SDS-PAGE-separated proteins onto nitrocellulose, and non-specific binding sites were blocked overnight at  $4^{\circ}\text{C}$  in PBS, pH 7.2 containing skimmed milk powder (2%, w/v), Tween 20 (0.1%, v/v). Primary antibodies, namely affinity-purified rabbit anti-GINR1 and monoclonal mouse anti- $\alpha$ -tubulin were diluted at 1:10 and 1:10000 in blocking solution, and blots were incubated overnight at  $4^{\circ}\text{C}$  on a horizontal shaker. Blots were then washed four times for 5 min in PBS, pH 7.2, containing 0.1% Tween 20 (PBS/Tween). The secondary antibodies, Alexa Fluor 680-conjugated goat anti-rabbit IgG and IRDye 800-conjugated goat anti-mouse IgG (Li-Cor, Lincoln, NE, USA) were diluted 1:5000 in Odyssey blocking solution (Li-Cor), and added to the respective blots. Blots were then incubated for 1 h at room temperature in the dark, followed by two washes with PBS/Tween and two washes with PBS, pH 7.2, prior to scanning in an Odyssey Infrared Imaging System (Li-Cor). All antibodies were stored at  $4^{\circ}\text{C}$  in the dark and could be reused several times.

### Determination of drug susceptibility in *G. lamblia*

Cultures with confluent trophozoite layers were incubated on ice for 15 min. Suspended motile trophozoites were counted and identical

**Table 1.** Overview of primers used in this study

Name	Sequence	Gene (accession number)	Construct (5'–3')
NRexpF	GATCTAGACCACAATAACGCCTTAATTACAGGCGCCCAGAT TTTAAATATGGTTGAAGGTTATCCTG	nitroreductase Fd-NR2 (ED080257; replaced EAA43030.1)	GA+XbaI+GDH promoter (100%)+NR 5' (CDS 1–19)
NRexpR	GATTAATTAATCACGCGTAGTCTGGGACATGGTATGGGTACTT TAAATGTAATGTCGAC		GA+PacI+stop+HA-Tag+NR (CDS 730–751)
NRquantF	CCTGCTGACAAGGCCGCA		CDS 526–794 plus 8 of 3' utr
NRquantR	AACACCAATTACTTAAATGTAATG		
ACTquantF	ACATATGAGCTGCCAGATGG	actin ACT (EAA39190)	CDS 715–933
ACTquantR	TCGGGGAGGCTGCAAAC		
HATagR	CGTAGTCTGGGACATGGTAT	see NRexpR	HA sequence 6–25

utr, untranslated region.

Please note that the nitroreductase GINR1 is now annotated as nitroreductase Fd-NR2. We use GINR1 in order to be in line with our previous publications.

numbers of trophozoites were inoculated into 96-well plates (0.2 mL per well) in the presence of compound or a solvent control (DMSO). The plates were incubated under anaerobic conditions (100% N<sub>2</sub>) at 37°C for 72 h, and living trophozoites were determined by the resazurin (Alamar blue) vitality assay as described previously.<sup>16,21</sup> The MIC of a compound was the concentration at which viable trophozoites could not be detected anymore.

### Determination of drug susceptibility in *E. coli*

Drug susceptibility of recombinant *E. coli* BL21(D3) lines expressing either GINR1 or GusA was tested by a conventional disc-diffusion agar procedure.<sup>22–24</sup> For this purpose, bacteria were grown to stationary phase (OD<sub>600</sub>=1) in Luria–Bertani (LB) medium containing 100 µg/mL ampicillin, and 0.3 mL of suspension was streaked on LB agar plates containing 100 µg/mL ampicillin. Whatman filter discs (5 mm diameter) were soaked with 7 µL of nitazoxanide or metronidazole stock solution (100 mM). Albendazole (100 mM) and kanamycin (2 mM) were included as negative and positive controls, respectively. The discs were air-dried for 5 min and placed on the plates. The plates were incubated under aerobic or microaerobic (5% O<sub>2</sub>/10% CO<sub>2</sub>/85% N<sub>2</sub>) conditions at 37°C for 24 h. Then, growth inhibition zone diameters were measured and the inhibition zone around the disc was calculated (in mm<sup>2</sup>).

### RNA analysis and quantification of expression by real-time RT–PCR

For quantification of GINR1 expression by real-time RT–PCR, trophozoites were grown until confluence as described above. Cells were harvested as described above, and RNA was extracted using a Qiagen RNeasy Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA was eluted with RNase-free water and stored at –80°C. First-strand cDNA was synthesized using a Qiagen OmniscriptRT Kit (Qiagen). After quantitative RT–PCR, expression levels were given as relative values in arbitrary units relative to the amount of actin. The primers NRquantF and NRquantR (Table 1) were used for the quantification of endogenous GINR1 expression, forming a PCR product of coding sequence 526–794 plus 8 nt of the untranslated region.<sup>12</sup> The primers NRquantF and HATagR (Table 1) were used for the quantification of exogenous GINR1 expression. The primers ACTquantF and ACTquantR (Table 1) were used for the quantification of a constitutively expressed gene, namely the *G. lamblia* actin transcript.<sup>12</sup>

Quantitative RT–PCR was carried out on a LightCycler™ Instrument (Roche Diagnostics, Rotkreuz, Switzerland) using SYBR Green (Qiagen) as a double-stranded DNA-specific fluorescent dye and continuous fluorescence monitoring as described previously.<sup>25</sup> All PCRs were performed in triplicate. PCR was started by initiating the 'Hot-Start' Taq DNA polymerase reaction at 95°C (15 min). Subsequent DNA amplification was carried out 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 during the temperature shift after each annealing phase in the 'single' mode. Fluorescence signals from the amplification products were quantitatively assessed by applying the standard software (version 3.5.3) of the LightCycler Instrument. Quantification of PCR products was performed during the log phase of the reaction and was achieved by using the secondary derivative maximum mode for plotting of the fluorescence signals versus the cycle numbers. Respective mean values from triplicate determinations were taken for the calculation. From the quantitative RT–PCR, mean values (±SEM) from triplicate determinations were assessed and expression levels of the GINR1 gene were given as values in arbitrary units relative to the amount of constitutively expressed 'housekeeping' gene actin.

### Statistical methods

For statistical analysis of the results from the drug treatment assays with *G. lamblia* and *E. coli* and from the determination of GINR1 expression levels, the Student's *t*-test in the GraphPad Prism 5 program (GraphPad Software, San Diego, CA, USA) was applied. Differences exhibiting *P* values of <0.05 or <0.001 were considered significant or highly significant, respectively. IC<sub>50</sub> values were calculated as described previously.<sup>16</sup>

## Results

### Expression of recombinant GINR1 in *G. lamblia* strain WBC6

In order to elucidate the importance of nitroreductase for susceptibility to nitro drugs, *G. lamblia* WBC6 was transfected with either GINR1 or GusA as a control, both under control of a constitutive promoter. After selection of stable transformants, the mRNA levels of recombinant GINR1 were determined by quantitative RT–PCRs specific for endogenous and recombinant GINR1. The corresponding protein expression levels were assessed by western blotting. Endogenous GINR1 mRNA levels were unchanged in WBC6–GINR1 as compared with WBC6–GusA. mRNA levels of recombinant GINR1 and endogenous GINR1 were three times higher than those of endogenous GINR1 alone. As shown by immunoblot, enhanced expression of GINR1 was also clearly visible at the protein level (Figure 1a and b).

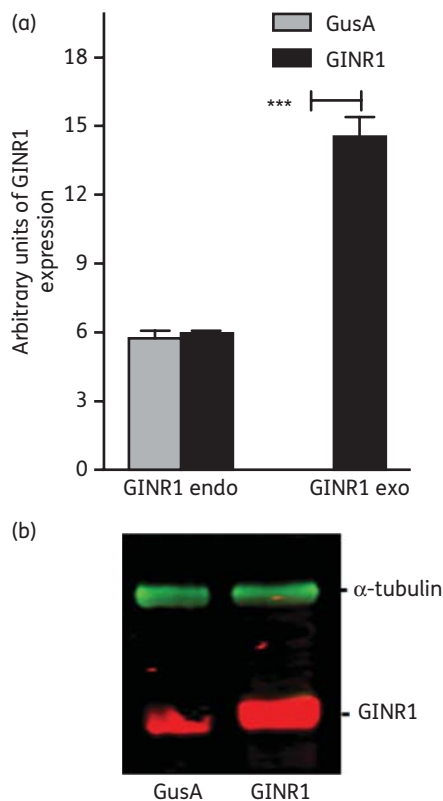
### WBC6–GINR1 is more susceptible to nitro drugs than WBC6–GusA

WBC6–GINR1 and WBC6–GusA were grown in the presence of either of the two nitro drugs nitazoxanide and metronidazole, and albendazole as a control. WBC6–GINR1 was more susceptible to the two nitro drugs than WBC6–GusA with regard to the corresponding IC<sub>50</sub> values (Table 2) and particularly at concentrations between 1.56 and 6.25 µM metronidazole and 3.13 µM nitazoxanide. These values have asterisks in the figure. In contrast, albendazole inhibited growth of both lines identically (Figure 2).

### *E. coli* expressing recombinant GINR1 exhibit increased susceptibility to nitazoxanide under semi-aerobic growth conditions

The previous results suggested that GINR1 activated rather than inactivated nitro drugs, and thus increased their efficacy. It could, however, not be ruled out that this effect was due to unknown functions of nitroreductases in metabolic pathways specific to *Giardia*.

To test whether the effect observed above in *Giardia* could be repeated in a completely different cellular environment, we generated recombinant *E. coli* lines producing either GINR1 or heterologous control enzyme GusA. In order to verify the expression of the recombinant proteins, immunoblot analyses of *E. coli* transformed with GINR1 and GusA were performed. *E. coli* transformed with GINR1 produced the recombinant protein even under non-induced conditions. Cross-reaction of the anti-GINR1 antibody with endogenous *E. coli* proteins was not observed (Figure 3a). Since overproduction of both GINR1 and control protein GusA in IPTG-induced *E. coli* strongly



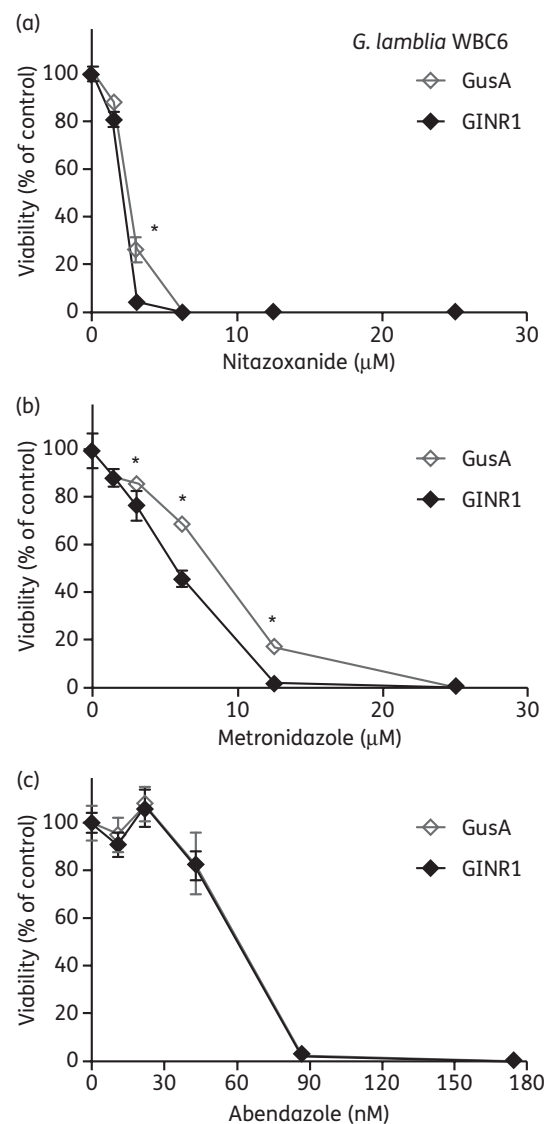
**Figure 1.** Overexpression of *G. lamblia* nitroreductase (GINR1) in transgenic *G. lamblia* WBC6. (a) Quantification of GINR1 mRNA levels by RT-PCR. Trophozoites were grown to confluence. RNA was extracted and reverse transcribed into cDNA. Endogenous (GINR1 endo) and chimeric GINR1 plasmid-encoded, exogenous (GINR1 exo) transcripts of GINR1 were quantified in relation to actin. Means  $\pm$  SEM are given for triplicates. Three asterisks indicate that values were different at a highly significant level,  $P < 0.01$ , as determined by Student's *t*-test. (b) Western blot showing GINR1 in control (GusA) and GINR1-overexpressing (GINR1) trophozoites. Trophozoites were grown to confluence, harvested and processed for SDS-PAGE. Positions of bands corresponding to endogenous  $\alpha$ -tubulin reference protein and GINR1 are indicated. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

**Table 2.** IC<sub>50</sub> values of the drug susceptibility assays presented in Figure 2

Drug	<i>G. lamblia</i> GusA, $\mu$ M (SEM)	<i>G. lamblia</i> GINR1, $\mu$ M (SEM)
Metronidazole	4.5 (0.9)	2.3 (0.5)
Nitazoxanide	1.5 (0.3)	1.1 (0.2)
Albendazole	0.04 (0.01)	0.04 (0.01)

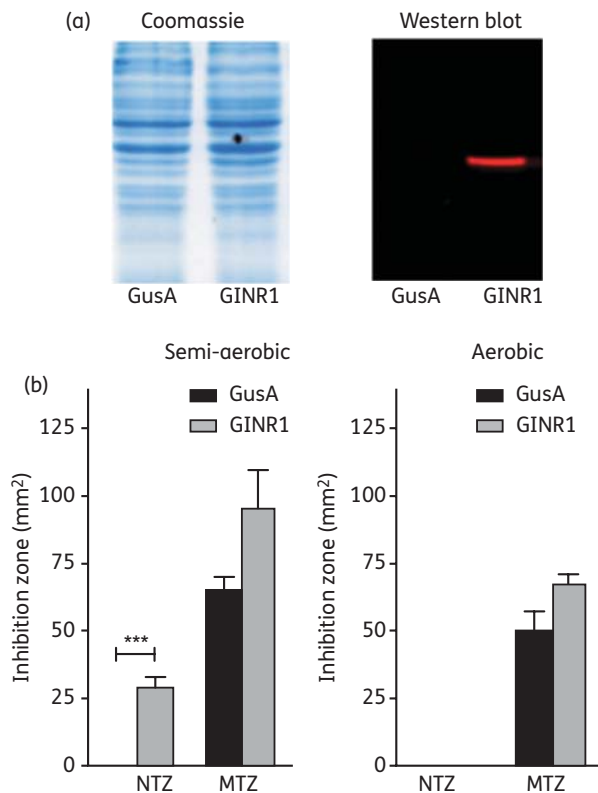
reduced growth, non-induced, recombinant *E. coli* cultures were chosen for growth inhibition assays under aerobic and semi-aerobic growth conditions.

Under aerobic as well as semi-aerobic growth conditions, nitazoxanide did not affect growth of control bacteria. Under semi-aerobic conditions, bacteria expressing GINR1 were significantly



**Figure 2.** Drug susceptibility of *G. lamblia* WBC6 expressing *E. coli* GusA and *G. lamblia* nitroreductase (GINR1). 96-well plates were inoculated with  $2 \times 10^2$  GusA or GINR1 trophozoites per well and grown in the presence of nitazoxanide (a), metronidazole (b) or albendazole (c) at various concentrations. After 72 h, growth of cells was monitored by a vitality assay based on the reduction of resazurin (Alamar blue) to a pink product that was assayed fluorimetrically. Mean values of the relative fluorescence units normalized to the control values ( $\pm$ SEM) are given for four replicates. Asterisks indicate significant differences between GusA and GINR1 trophozoites (paired *t*-test, two-sided,  $P < 0.05$ ).

more susceptible to nitazoxanide than control bacteria. However, under aerobic conditions, nitazoxanide had no inhibitory effect (Figure 3b). Interestingly, metronidazole inhibited growth of control or of GINR1-transformed bacteria even under aerobic conditions. Bacteria expressing GINR1 exhibited a slightly more pronounced inhibition under semi-aerobic conditions (Figure 3b). Kanamycin inhibited both transformed lines similarly with inhibition zones of  $\sim 150$  mm<sup>2</sup> in both cases. Albendazole did not affect growth in either line. No significant differences in

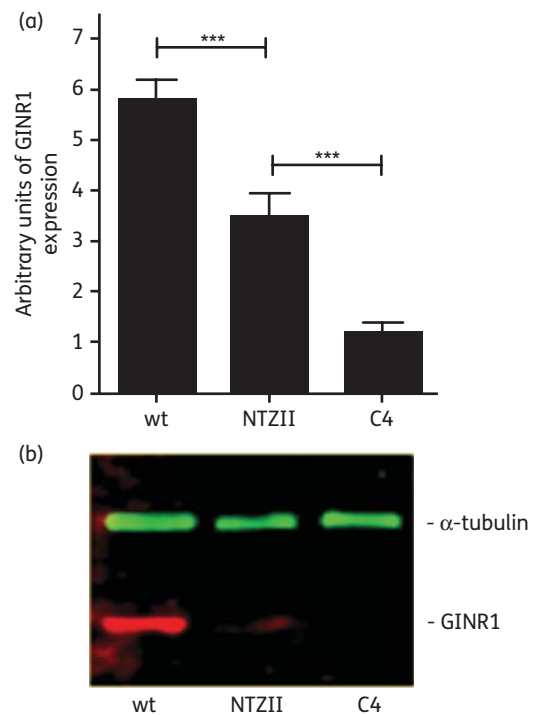


**Figure 3.** Susceptibility of *E. coli* BL21, expressing GusA or GINR1 to nitazoxanide (NTZ) and metronidazole (MTZ). (a) Coomassie-stained gel (loading control) and corresponding western blot showing the expression of GINR1 in GINR1-transformed *E. coli*. (b) Drug susceptibility assays. Lines were plated, discs containing the drugs were added as described and plates were incubated under aerobic or semi-aerobic (5% O<sub>2</sub>/10% CO<sub>2</sub>/85% N<sub>2</sub>) conditions. After 24 h, diameters of inhibition zones were determined and surface areas of inhibition zones were calculated. Means  $\pm$  SEM are given for three replicates. Three asterisks indicate that values were different at a highly significant level (paired *t*-test, one-sided,  $P < 0.001$ ). This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

susceptibility to nitro drugs between the two recombinant *E. coli* lines were detected when they were grown aerobically.

### Nitazoxanide-resistant *Giardia* have lower GINR1 expression levels

As shown above, overexpression of GINR1 is correlated with a higher susceptibility to nitro drugs. Unfortunately, we could not show the opposite, i.e. a lower susceptibility in nitroreductase-silenced cells since expression of antisense constructs under the same conditions as described above were lethal in our hands, and trophozoites expressing a GINR1-antisense construct under a weaker promoter were so severely impaired in growth that drug susceptibility assays could not be performed (data not shown). Therefore, we induced resistance to nitazoxanide in WBC6 as described earlier for the previously characterized line derived from clone C4<sup>12</sup> and obtained a nitazoxanide-resistant line (NTZII) independently of clone C4. MIC values for both lines were  $>100 \mu\text{M}$  as compared with  $25 \mu\text{M}$  for the wild-



**Figure 4.** Differential expression of *G. lamblia* nitroreductase (GINR1) in *G. lamblia* WBC6 and drug-resistant derivatives of WBC6. (a) Quantification of GINR1 mRNA levels by RT-PCR. Trophozoites were grown to confluence. RNA was extracted and reverse transcribed into cDNA. Transcripts of GINR1 were quantified in relation to actin. Means  $\pm$  SEM are given for triplicates. Three asterisks indicate that values were different at a highly significant level,  $P < 0.001$ , as determined by Student's *t*-test. (b) Western blot showing GINR1 in wild-type WBC6 (wt), WBC6 grown on successively increasing nitazoxanide concentrations (strain NTZII) and the previously described nitazoxanide/metronidazole-resistant clone C4 (C4). Trophozoites were grown to confluence, harvested and processed for SDS-PAGE. Positions of bands corresponding to endogenous  $\alpha$ -tubulin reference protein and GINR1 are indicated. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

type. The relative mRNA levels of GINR1 in clone C4 were  $>4.9$  times lower than in WBC6, and also significantly lower than in line NTZII (Figure 4a). The lower expression of GINR1 in nitazoxanide-resistant lines was also shown to occur at the protein level (Figure 4b).

### Discussion

Metronidazole and other antiparasitic nitro drugs are considered to be prodrugs, which are activated by partial reduction forming a toxic radical<sup>26</sup> or a partially reduced nitroso or hydroxylamine intermediate.<sup>13</sup> On the other hand, complete reduction detoxifies nitro compounds, and thus allows some bacteria to use highly toxic compounds such as trinitrotoluene as carbon sources.<sup>27</sup> Overexpression of target enzymes yielding toxic radicals would then correlate with higher susceptibility, while overexpression of target enzymes performing total reduction would result in increased resistance. Our results

presented above suggest that in *Giardia*, the nitroreductase GINR1 is one of the enzymes with the ability to perform partial reduction of nitro compounds: overexpression of GINR1 in *G. lamblia* is followed by higher susceptibility to both metronidazole and nitazoxanide. Interestingly, GINR1 has a ferredoxin domain, which nicely corroborates the findings about the involvement of ferredoxin as an electron donor for the reduction of metronidazole and other nitro compounds in *Giardia*. Recent findings have shown that PFOR is not the only possible electron donor for this reduction.<sup>28</sup> Our findings suggest that GINR1 is another donor, thus explaining resistance formation without involvement of the PFOR system.

However, since trophozoites transformed with antisense constructs are not viable, it cannot be ruled out that GINR1 carries out some still unknown, but essential, functional activities in the intermediate metabolism of *Giardia*. Therefore, the results obtained with *E. coli* are of particular interest since they indicate that GINR1 works in a different cellular environment in the same way as in *Giardia* (at least with respect to nitro drugs). Moreover, since *E. coli* grow well under aerobic as well as under semi-aerobic conditions, it is possible to explore the effects of these conditions on GINR1. *E. coli* that express GINR1 are susceptible to nitazoxanide under semi-aerobic conditions, but not under aerobic conditions. This corroborates our previous findings that recombinant GINR1 does not reduce nitazoxanide *in vitro* under aerobic conditions.<sup>11</sup> The situation is quite different in the presence of metronidazole. Under both aerobic and semi-aerobic conditions, the two *E. coli* lines are clearly susceptible to metronidazole, with only slight enhancement in *E. coli* expressing GINR1. This indicates that in *E. coli* reduction of metronidazole, but not of nitazoxanide, may be due to endogenous *E. coli* nitroreductases expressed even under aerobic growth conditions, thus by metabolic pathways different from PFOR or GINR1-homologous nitroreductases, since both systems are absent from the *E. coli* genome. In this context it is noteworthy to mention that most of the literature on the antibacterial effects of metronidazole and related nitro drugs concern anaerobic or semi-aerobic bacteria such as *Helicobacter pylori*. Since *E. coli* obviously is susceptible to metronidazole, it represents an ideal model of choice to study this drug. This susceptibility has been reported earlier by other authors.<sup>24</sup> Several nitroreductases have been identified in the *E. coli* genome. Of these, the *Ntr* gene is most interesting, since transfection of mammalian tumour cells and expression of *Ntr* rendered these cells metronidazole sensitive.<sup>29,30</sup> Another interesting candidate is NfsA, a nitroreductase with a good affinity to nitrofurazones and other nitroaromatic compounds.<sup>31</sup>

These results, and the fact that nitazoxanide and metronidazole resistance in *G. lamblia* correlated with strongly reduced GINR1 expression, suggested participation of the nitroreductase GINR1 in activation of nitro drugs. Thus, the expression level of this enzyme clearly represents a key factor for the determination of susceptibility versus resistance to nitro drugs in *G. lamblia*. However, previous studies on differential gene expression patterns in wild-type and resistant lines have shown that the expression levels of a variety of genes are affected. This indicated that resistance formation takes place on a multigenic rather than a monogenic level.<sup>32</sup> Further studies will be necessary to provide coherent information on the complex pathways that determine the anti-giardial activity of nitro compounds.

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## Transparency declarations

None to declare.

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