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Metabolism of nitro drugs metronidazole and nitazoxanide in *Giardia lamblia*: characterization of a novel nitroreductase (GlNR2)

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Objectives: The protozoan parasite *Giardia lamblia* causes giardiasis, a persistent diarrhoea. Nitro drugs such as the nitroimidazole metronidazole and the nitrothiazolide nitazoxanide are used for the treatment of giardiasis. Nitroreductases may play a role in activating these drugs. *G. lamblia* contains two nitroreductases, GlNR1 and GlNR2. The aim of this work was to elucidate the role of GlNR2.

Methods: Expression of GlNR2 was analysed by reverse transcription PCR. Recombinant GlNR2 was overexpressed in *G. lamblia* and drug susceptibility was analysed. Recombinant GlNR2 was subjected to functional assays. *Escherichia coli* expressing full-length or truncated GlNR1 and GlNR2 were grown in the presence of nitro compounds. Using *E. coli* reporter strains for nitric oxide and DNA damage responses, we analysed whether GlNR1 and GlNR2 elicited the respective responses in the presence, or absence, of the drugs.

Results: *G. lamblia* trophozoites overexpressing GlNR2 were less susceptible to both nitro drugs as compared with control trophozoites. GlNR2 was a functional nitroreductase when expressed in *E. coli*. *E. coli* expressing GlNR1 was more susceptible to metronidazole under aerobic and semi-aerobic and to nitazoxanide under semi-aerobic growth conditions. *E. coli* expressing GlNR2 was not susceptible to either drug. In reporter strains, GlNR1, but not GlNR2, elicited nitric oxide and DNA repair responses, even in the absence of nitro drugs.

Conclusions: These findings suggest that GlNR2 is an active nitroreductase with a mode of action different from that of GlNR1. Thus, susceptibility to nitro drugs may depend not only on activation, but also on inactivation of the drugs by specific nitroreductases.

Keywords: drug susceptibility, mode of action, drug targets

Introduction

Giardia lamblia (syn. *Giardia duodenalis*; *Giardia intestinalis*), a flagellated protozoan, is the most common causative agent of persistent diarrhoea worldwide.[1,2] For antigiardial chemotherapy, some effective drugs are available, namely the nitroheterocyclic drugs tinidazole, metronidazole, furazolidone, the acridine derivative quinacrine, the aminoglycoside paromomycin, and the benzimidazole albendazole.[3,4] In 2000, the nitrothiazolide nitazoxanide was introduced as an alternative option.[5]

According to a widely accepted hypothesis, metronidazole and other related nitro drugs are reduced to a nitro radical by electrons coming from the enzyme pyruvate:flavodoxin/ferredoxin oxidoreductase (PFOR), a protein lacking in higher eukaryotic cells.[6,7] According to this model, PFOR transfers electrons from pyruvate to ferredoxin. The resulting reduced ferredoxin is then reoxidized by ferredoxin:NAD oxidoreductase transferring its electrons to NAD(P). The resulting NAD(P)H may then transfer its electrons to O_2, a reaction catalysed by NAD(P)H oxidase.[7] In this model, nitro drugs may capture electrons directly from the reduced ferredoxin or from the NAD(P)H oxidase, thus yielding toxic radicals causing irreversible damage in the parasite. Another line of evidence for PFOR as a main target for nitro drugs in *Giardia* has been obtained in metronidazole-resistant isolates, some of which exhibited lower PFOR expression levels.[8]

In the last decade, however, evidence has emerged that PFOR may not be the only target of nitro drugs in semi-aerobic or anaerobic pathogens. Recent findings in *Trichomonas vaginalis* suggest that metronidazole and other nitroimidazoles covalently bind and thereby inactivate proteins related to the thioredoxin reductase pathway. Resistant cells overcome this blocking by reregulating other enzymes involved in oxidoreductive processes, such as PFORs. In this model, down-regulation of PFOR would be a consequence rather than a prerequisite of resistance formation.[9] Moreover, recent studies on *Giardia* clones and strains resistant against nitro drugs have revealed that resistance may

occur without down-regulation of PFOR.[10,11] Therefore, it is likely that there are alternative targets for nitro drugs, besides PFOR. Although some nitro drugs seem to interact directly with PFOR, it is unlikely that ferredoxin directly performs the reduction of the nitro group. It is more probable that this reaction is catalysed by nitroreductases.[12] Nitroreductases are present in many archaebacteria and eubacteria, where they allow the assimilation of nitro compounds as carbon sources.[13,14] Interestingly, nitroreductases are also found in the genomes of microaerophilic or anaerobic eukaryotic parasites, such as *Entamoeba histolytica* and *G. lamblia*, which may have acquired them from prokaryotes by lateral transfer.[15]

The genome of *G. lamblia* (clone WBC6) contains two genes encoding nitroreductases, namely GlNR1 (accession no. EDO80257) and GlNR2 (accession no. XM_764091.1). Whereas GlNR1 has been characterized to some extent by our group,[11,16,17] detailed information concerning GlNR2 is lacking. The polypeptide sequence of GlNR2 is highly similar to that of GlNR1. Both proteins possess a ferredoxin domain with four Fe-S clusters at their N terminus and a nitro-flavin mononucleotide (FMN) reductase domain at their C terminus (Figure 1).

The potential for employing molecular genetic approaches in *Giardia*, such as complementation of mutants, is very limited. In addition, *Giardia* is restricted to anaerobic growth in a complex medium, thus further narrowing its experimental potential. *Escherichia coli*, however, grows under aerobic as well as under semi-aerobic conditions in complex and defined media, and offers a variety of molecular genetic tools, such as reporter systems suitable for the heterologous expression and characterization of nitroreductases. NorR is a σ^{54}-dependent transcription factor with a ferrous iron centre binding nitric oxide followed by the activation of transcription of the *norVW* operon.[18] The *norV* promoter can be fused to *lacZ*, thus creating a suitable reporter strain for nitric oxide stress.[19] The reporter strain SOS-R1 is deficient in major nitroreductases[20,21] and carries a null mutation in the multidrug efflux *tolC* gene, increasing susceptibility to various drugs and antibiotics. Furthermore, this strain contains a fusion between the SOS-inducible *sfiA* promoter and the *lacZ* reporter gene and is thus suitable for a β-galactosidase-based chromometric detection of antimicrobial compounds affecting DNA replication.[22] By introducing a heterologous nitroreductase in this background, it is possible to investigate whether this nitroreductase activates nitro compounds inducing DNA damage.

Here, we present the characterization of GlNR2 by heterologous expression in *G. lamblia* and *E. coli*, and show that GlNR2 is a functional nitroreductase with properties different from GlNR1.

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 (kindly provided by Ch. Leumann). Nitroimidazole compound C17 was kindly provided by J. A. Upcroft (Molecular Genetics Laboratory, Queensland Institute of Medical Research, Brisbane, Australia). Nitazoxanide, metronidazole, C17 and albendazole were kept as 100 mM stock solutions in DMSO at −20°C.

Axenic culture of Giardia trophozoites

Trophozoites from *G. lamblia* clone WBC6 as well as clones 713 and 106[23] were grown under anaerobic conditions in 10 mL culture tubes (Nunc, Roskilde, Denmark) containing modified TYI-S-33 medium. The nitazoxanide-resistant clone C4[11] was maintained in the presence of 40 μM nitazoxanide, the metronidazole-resistant clones C5,[11] 713-M3 and 106-2ID10[23] in the presence of 40 μM metronidazole, and the C17-resistant clones 713-M3-C17 and 106-17A[23] in the presence of 15 μM C17. Twenty-four hours prior to an experiment, the 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 × 10e4 trophozoites to a new culture tube. Trophozoites were grown to near confluence and harvested by centrifugation (600 **g**, 15 min, 4°C). Trophozoite pellets were washed three times with PBS, pH 7.2 and stored at −80°C.

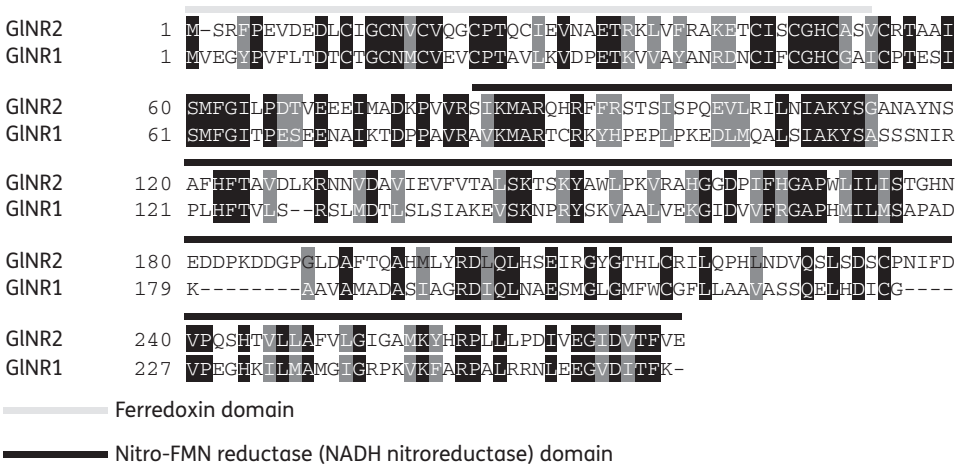

Figure 1. Alignment of GlNR2 (XM_764091.1) with the previously characterized nitroreductase GlNR1 (EDO80257). The alignment was produced with ClustalW and edited with Boxshade. The conserved ferredoxin and nitro-FMN reductase domains are highlighted.

Overexpression of recombinant GlNR2 in G. lamblia

Clones with high expression levels of the recombinant proteins were used for the recloning of the GlNR1,[17] GlNR2 (this study) and GusA (as a control)[17,24] 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 and taking into account that the coding sequence of GlNR2 contained an XbaI site, a forward primer was designed starting with an NheI site followed by the constitutive glutamate dehydrogenase promoter. NheI creates an XbaI isoschizomer, thus allowing cloning into an XbaI site. In the reverse primer, a sequence encoding a human influenza haemagglutinin tag was included upstream of the PacI site (Table S1, available as Supplementary data at *JAC* Online). PCRs were performed using the *pfu* polymerase (Promega, Madison, WI, USA) and fragments were cloned into the Zero Blunt TOPO vector (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Inserts were excised with NheI and PacI and ligated into pPacV, thus yielding pPacV-GlNR2. Transfection and selection of transgenic trophozoites were performed as previously described.[25]

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 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_2) at 37°C for 72 h and living trophozoites were determined by the resazurin vitality assay as described previously.[17]

Overexpression of recombinant GlNR1 and GlNR2 in E. coli

For expression in BL21(DE3), cloning of GlNR1,[16,17] GlNR2 (this study) and GusA as a control[17,24] as well as truncated GlNR1 and GlNR2 without ferredoxin domains (this study) in the *E. coli* His-tag expression vector system pET151 (pET151 directional TOPO; Invitrogen) was performed according to the manufacturer's instructions. For expression of GlNR1, GlNR2 and GusA in *E. coli* strains SOS-R1 (carrying on the chromosome *sfiA* promoter fused to *lacZ*; kindly provided by D. F. Ackerley, School of Biological Sciences, Victoria University of Wellington, Wellington, New Zealand)[22] and RK4353/pP*norV-lacZ*-1 (carrying on the plasmid *norV* promoter fused to *lacZ*; *E. coli* strain was kindly provided by L. M. Saraiva, Instituto de Investigação, Centifica Tropical, Oeiras, Portugal; origin of plasmid, see below),[19] the expression plasmid pUCX[22] (also kindly provided by D. F. Ackerley) was used. This vector contained the ampicillin resistance marker for selection of transformants and allowed the expression of recombinant proteins under the control of the strong, IPTG-inducible *tac* promoter (P*tac*). P*tac* was regulated by a *lac* repressor that is encoded by the *lacI*^{q} gene located on the same plasmid.[22] Recloning of the GlNR1,[16] GlNR2 (this study) and GusA[17,24] open reading frames into pUCX provided plasmids pGlNR1x, pGlNR2x and pGusAx, respectively, and was achieved by exactly following an inverse fusion PCR cloning strategy, as recently described.[26] Furthermore, plasmid pAA182-P*norV*[19] (kindly provided by L. M. Saraiva), containing an ampicillin resistance marker, was used as PCR template for recloning of the *PnorV:lacZ* fusion in the pCR-Blunt II TOPO vector (Invitrogen) containing a kanamycin resistance marker. This recloning step provided a plasmid construct, pP*norV-lacZ*-1, suitable for subsequent transformation of ampicillin-resistant RK4353/pGlNR1x, pGlNR2x and pGusAx strains by selection for ampicillin (100 μg/mL)/kanamycin (50 μg/mL) double-resistant clones. The *E. coli* strains and plasmids used in this study are listed in Table S1, available as Supplementary data at *JAC* Online; the primers for the corresponding PCR reactions are listed in Table S2, available as Supplementary data at *JAC* Online.

Purification and functional assay of GlNR2

For His-tag affinity purification, the coding sequence of GlNR2 was cloned into pET-His151 (see above). Overexpression was achieved in BL21(DE3). His-tag purification and nitroreductase assays were performed as previously described for GlNR1.[16]

Determination of drug susceptibility in E. coli

Drug susceptibility of recombinant *E. coli* BL21(DE3) lines expressing either GlNR1, GlNR2 or GusA were tested by a conventional disc-diffusion agar procedure as described previously.[17] For this purpose, bacteria were grown to stationary phase ($OD_{600}=1$) in Luria–Bertani (LB) medium containing 100 μg/mL ampicillin and 0.5 mM IPTG, 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 solutions (100 mM). The discs were air-dried for 5 min and then placed on the plates. The plates were incubated under aerobic or semi-aerobic (5% O_2, 10% CO_2, 85% N_2) 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^2).

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

For quantification of gene 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 the 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 the QIAGEN Omniscript RT Kit (QIAGEN, Hilden, Germany). Quantitative RT–PCR was performed as described before.[17] The primers used for quantitative RT–PCR are given in Table S1, available as Supplementary data at *JAC* Online. From the quantitative RT–PCR, mean values (±SE) from triplicate determinations were assessed and expression levels of the GlNR1 gene were given as values in arbitrary units relative to the amount of constitutively expressed actin.

β-Galactosidase assay in E. coli reporter strains

Liquid cultures of *E. coli* reporter strains SOS-R1 and RK4353/pP*norV-lacZ*-1-carrying plasmids with the nitroreductases GlNR1, GlNR2 or GusA as a control (see Table S1, available as Supplementary data at *JAC* Online) were grown to early exponential phase (OD_{600} ~0.2–0.3) and treated with metronidazole, nitazoxanide (1 μM) or DMSO as a solvent control for an additional 150 min. Then, the OD_{600} was determined and β-galactosidase activity was assayed as previously described.[27] Briefly, 50 μL of each culture was added to 1 mL of PBS buffer containing 50 mM mercaptoethanol, 20 ppm SDS and 40 μL of chloroform. After vortexing and phase separation of the chloroform, 0.1 mL was added to the wells of a 96-well plate. Then, 0.1 mL of PBS containing 1 mM chlorophenyl red-β-galactoside (Roche, Basel, Switzerland) was added; absorption at 570 nm was read at various timepoints and the initial velocity (ΔA_{570}/min) was determined. Blanks (buffer without cells) were included and subtracted.

Statistical methods

Statistical analysis of the results was performed with suitable tools from the open-source software package R.[28] Differences exhibiting *P* values of

<0.01 were considered significant. 50% Inhibitory concentration (IC_{50}) values were calculated after the logit-log transformation of the relative growth (RG; control=1) according to the formula $\ln[RG/(1-RG)]=a\times \ln(\text{drug concentration})+b$ followed by regression analysis.

Results

mRNA levels of the nitroreductases GlNR1 and GlNR2 in metronidazole-resistant G. lamblia strains

In order to compare nitroreductase mRNA levels of strains resistant to nitro compounds, two resistant strains of WBC6 were grown until confluence and the expression levels of the GlNR1 and GlNR2 transcripts were determined in relation to actin as a housekeeping gene. Moreover, we included strains derived from isolates 106 and 713 in our study. These strains are resistant to metronidazole and to the 5-nitroimidazole C17.[23] The phenotypes of all strains were confirmed prior to our investigations. In the WBC6 background, the mRNA levels of GlNR1 were significantly lower in a nitazoxanide- and metronidazole-resistant strain, clone 4 (C4), and a metronidazole-resistant strain, clone 5 (C5). In the 106 background, GlNR1 levels were significantly lower in a strain resistant to the nitro compound C17. In the 713 background, both strains resistant to metronidazole and to C17 had lower GlNR1 levels. Conversely, GlNR2 mRNA levels remained unchanged in both WBC6-derived strains, C4 and C5, and were significantly higher in the metronidazole-resistant strains derived from 106 and 713 (Figure 2). It is, however, noteworthy that the expression levels of both nitroreductases were around one magnitude lower in the strains 106 and 713 than in WBC6. Taken together, the expression profile of GlNR2 was clearly different from that of GlNR1.

WBC6-GlNR2 is less susceptible to metronidazole than WBC6-GusA

In a previous study, we overexpressed GlNR1 in *G. lamblia* WBC6 and observed an increased susceptibility to nitazoxanide and metronidazole.[17] In order to investigate whether overexpression of GlNR2 had similar effects, we overexpressed GlNR2 in *G. lamblia* WBC6. Overexpression in WBC6-GlNR2 turned out to be >10-fold in comparison with the WBC6-GusA control strain, as verified on the mRNA level by quantitative RT–PCR.

WBC6-GlNR2 and WBC6-GusA were grown in the presence of the two nitro drugs nitazoxanide or metronidazole, or albendazole as a control. WBC6-GlNR2 was less susceptible to metronidazole than WBC6-GusA, as indicated by the corresponding

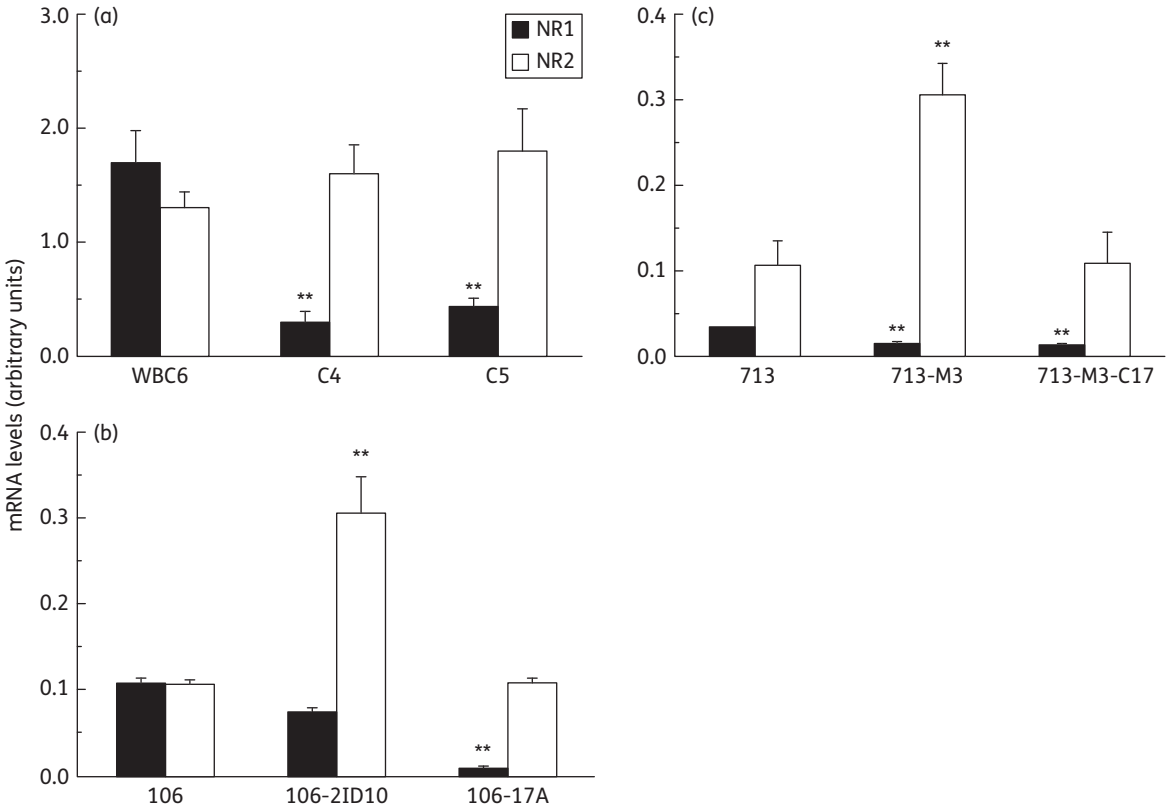

Figure 2. mRNA levels of *G. lamblia* nitroreductases GlNR1 (NR1, black columns) and GlNR2 (NR2, white columns) in different isolates of *G. lamblia* WBC6 and drug-resistant derivatives of these isolates. Trophozoites were grown to confluence. RNA was extracted and reverse transcribed into cDNA. Transcripts of GlNR1 were quantified in relation to actin. Mean values±SE are given for triplicates. Values marked by asterisks are significantly different from the control (paired *t*-test, two sided; **, $P<0.01$). (a) WBC6 and clones C4 (resistant to nitazoxanide and metronidazole) and C5 (resistant to metronidazole). (b) Strain 106 and derivatives 106-2ID10 (resistant for metronidazole) and 106-17A (resistant to C17). (c) Strain 713 and derivatives 713-M3 (resistant to metronidazole) and 713-M3-C17 (resistant to C17).

IC_{50} values. In contrast, nitazoxanide and albendazole inhibited the growth of both lines identically (Table 1).

Heterologous expression and functional analysis of GlNR2

The coding sequence of GlNR2 was amplified by PCR, cloned and expressed in *E. coli*. The recombinant protein was a major protein in IPTG-induced *E. coli* and could further be purified by His-tag affinity chromatography (Figure 3a). The purified protein was subjected to a nitroreductase assay based on the oxidation of NADH in the presence of dinitrotoluene. The recombinant protein readily reduced dinitrotoluene in a concentration-dependent manner (Figure 3b). When metronidazole was offered as a substrate, only a very low activity could be detected; when nitazoxanide was offered as a substrate, activity was not detectable (Figure 3c).

E. coli expressing recombinant GlNR2 exhibits decreased susceptibility to metronidazole

The previous results suggested that GlNR2 clearly differed from GlNR1. Whereas GlNR1 activated rather than inactivated nitro drugs, and thus increased their efficacy ([17] and this study), GlNR2 might act in the opposite direction. In order to investigate this hypothesis in more detail, our functional *in vitro* assay system was too limited. In particular, it could not discriminate between partial and complete reduction. Therefore, we generated recombinant *E. coli* lines producing GlNR1 and GlNR2 either as full-length enzymes or without ferredoxin domains, or the control enzyme GusA. Since overproduction of proteins in IPTG-induced *E. coli* strongly reduced growth, non-induced, recombinant *E. coli* cultures were chosen for growth inhibition assays under aerobic and semi-aerobic growth conditions, as described previously.[17]

Under aerobic as well as under semi-aerobic growth conditions, nitazoxanide did not affect the growth of the control bacteria. Under semi-aerobic conditions, bacteria expressing GlNR1 were significantly more susceptible to nitazoxanide than control bacteria, yielding inhibition zones of 30.8 ± 4 mm^2 versus <1 mm^2. Under aerobic conditions, nitazoxanide had no inhibitory effect on bacteria expressing GlNR1, with inhibition zones <1 mm^2. Inserts other than GlNR1 had no effect. In the presence of metronidazole, a completely different picture was observed. Bacteria transformed with GusA, GlNR1 or both nitroreductases without the ferredoxin domain were growth inhibited under aerobic and semi-aerobic conditions. Bacteria expressing GlNR1 exhibited a significantly higher inhibition than the others. Interestingly, bacteria expressing GlNR2 were inhibited neither under aerobic nor semi-aerobic growth conditions (Figure 4a and b). Thus, GlNR1 and GlNR2 clearly had antithetical effects in this system.

Table 1. Drug susceptibility of *G. lamblia* WBC6 expressing *E. coli* β-glucuronidase A (GusA) and of *G. lamblia* nitroreductase (GlNR2)

	IC_{50} μM (95% CI)	
Drug	*G. lamblia* GusA	*G. lamblia* GlNR2
Metronidazole	1.7 (1.4–2.1)	4.8 (3.5–6.5)
Nitazoxanide	0.7 (0.6–0.8)	0.7 (0.6–0.8)
Albendazole	0.03 (0.02–0.0)	0.04 (0.03–0.05)

Ninety-six-well plates were inoculated with 10^3 trophozoites of GusA or GlNR2 trophozoites per well and grown in the presence of metronidazole, nitazoxanide, or albendazole at various concentrations. After 72 h, the 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. IC_{50} values were determined after logit-log transformations of the data.

GlNR1 and GlNR2 differ with respect to nitric oxide and DNA repair responses in E. coli

The previous results suggested that GlNR1 and GlNR2 exhibited opposed functional properties with respect to eliciting nitric oxide and DNA damage responses in *E. coli*. To test this, their coding sequences were overexpressed in the *E. coli* reporter strains RK4353/pP*norV-lacZ*-1 (nitric oxide response) and SOS-R1 (DNA repair response). Strains overexpressing GusA were used as a control. The transformed bacteria were then exposed to metronidazole, nitazoxanide or DMSO as a solvent control for 150 min, harvested and β-galactosidase activity was assessed. In RK4353/pP*norV-lacZ*-1 bacteria expressing GlNR1, β-galactosidase activity was significantly ($P<0.01$) higher than in cells expressing GlNR2 or GusA, even in the absence of nitro drugs. This suggested that in cells expressing GlNR1, the nitric oxide response was constitutively switched on. The response was even more increased in the presence of metronidazole, indicating that this compound elicited a nitric oxide response in addition to the control (Figure 5a). The DNA repair response was significantly increased in cells expressing GlNR1 irrespective of the presence, or absence, of nitro compounds (Figure 5b). Cells expressing GlNR2 did not significantly differ from the control in any case.

Discussion

Metronidazole and other antiparasitic nitro drugs are considered to be prodrugs, which are activated by partial reduction forming a toxic radical,[29] or partially reduced nitroso or hydroxylamine intermediates[30] causing DNA damage.[31] 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.[32] The overexpression of target enzymes yielding toxic radicals would then correlate with higher susceptibility, while the overexpression of target enzymes performing a total reduction would result in increased resistance. Our results presented above suggest that *G. lamblia* expresses two nitroreductases with functional activities working in both directions. GlNR1 is one of the enzymes with the ability to partially reduce nitro compounds to toxic intermediates, since it was shown that overexpression of GlNR1 in *G. lamblia* is followed by higher susceptibilities to both metronidazole and nitazoxanide.[17] Conversely, strains resistant to nitazoxanide and/or metronidazole express GlNR1 at lower levels, as shown previously[11,17] and in this study. The observation that C17-resistant cell lines do not express higher levels of GlNR2

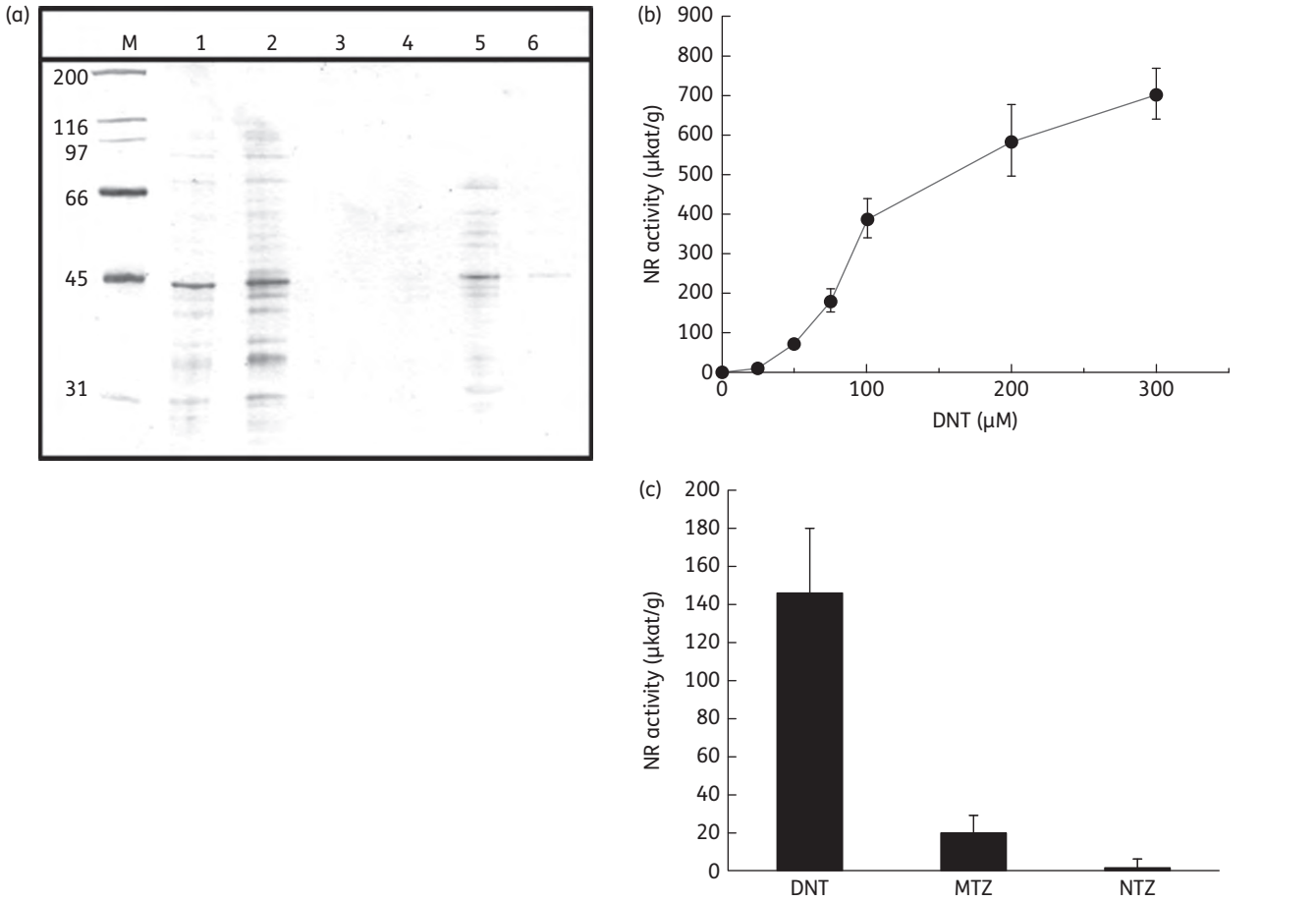

Figure 3. Heterologous expression in *E. coli* BL21(DE3) and functional assay of GlNR2. (a) SDS–PAGE of His-tag-purified recNR2. M, markers with sizes in kDa; lane 1, crude extract; lane 2, flow through; lane 3, wash; lane 4, wash with 10 mM imidazole; and lanes 5 and 6, fractions eluted with 200 mM imidazole. (b) Functional assay of fraction 5 in the presence of increasing concentrations of dinitrotoluene (DNT). (c) Functional assay in the presence of the nitro compounds DNT, metronidazole (MTZ) or nitazoxanide (NTZ, 50 μM each). Mean values ± SE are given for quadruplicates.

may be explained by the fact that the 5-nitroimidazole C17 is metabolized differently from other nitroimidazoles, including metronidazole.[23] The overexpression of GlNR2, however, does not affect the susceptibility to nitazoxanide, but decreases the susceptibility to metronidazole. In resistant strains, its expression levels remain unchanged or are even increased. These results suggest that GlNR2 detoxifies rather than activates nitro compounds, e.g. by complete reduction to less toxic amines.

The results obtained with *E. coli* are of particular interest, since they indicate that GlNR1 and GlNR2 work in a different cellular environment in the same way as in *Giardia* (at least with respect to nitro drugs). *E. coli* expressing GlNR1 and GlNR2 without a ferredoxin domain have no phenotypes different from the control, suggesting that this domain is essential for their activities. GlNR1 enhances the susceptibility to nitazoxanide under semi-aerobic conditions but not under aerobic conditions and to metronidazole under both growth conditions. GlNR2 decreases the susceptibility to metronidazole under both growth conditions. This corroborates our previous findings that recombinant GlNR1 does not reduce nitazoxanide *in vitro* under aerobic conditions[11] and our present *in vitro* results with the recombinant GlNR2. Moreover, our results suggest that in *E. coli* BL21(DE3), the strain used in these experiments, endogenous nitroreductases are expressed even under aerobic growth conditions, which is fully consistent with previously published results.[20,21,33] While these endogenous nitroreductases reduce metronidazole to toxic intermediates, GlNR2 detoxifies them, thus eliminating metronidazole toxicity against *E. coli*. The fact that *E. coli* BL21(DE3) is susceptible to metronidazole has been reported earlier.[17,34] These results can, however, not be extended to other *E. coli* strains, e.g. SOS-R1 is non-susceptible to metronidazole under aerobic as well as semi-aerobic growth conditions (data not shown).

The results with the *E. coli* reporter strains for nitric oxide response (RK4353/pAA182-P*norV*) and DNA damage (SOS-R1) confirm that GlNR1 and GlNR2 have a different mode of action and add an important novel facet. Even in the absence of nitro drugs, GlNR1 elicits responses in both systems whereas GlNR2 does not. This may be explained by a direct activation of the sensors in both systems or the presence of nitro compounds in the complex culture medium that are activated by GlNR1, but not by GlNR2. A good candidate may be nitrite,[35] which would be reduced to nitric oxide by GlNR1, but not by GlNR2.

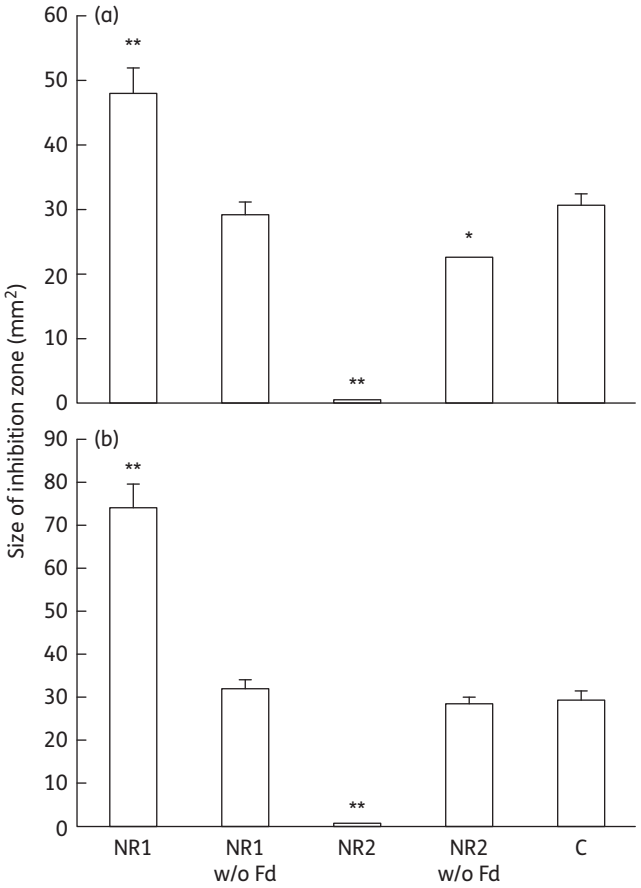

Figure 4. Susceptibility to metronidazole of *E. coli* BL21(DE3) expressing GusA as a control (C), GlNR1 (NR1) or GlNR2 (NR2) with or without ferredoxin domain (w/o Fd). Lines were plated, discs containing the drugs were added as described and plates were incubated under aerobic (a) or semi-aerobic (b) conditions. After 24 h, diameters of inhibition zones were determined and surfaces of inhibition zones were calculated. Mean values ± SE are given for triplicates. Values marked by asterisks are significantly different from the control (paired *t*-test, two sided; *, $P<0.05$; **, $P<0.001$).

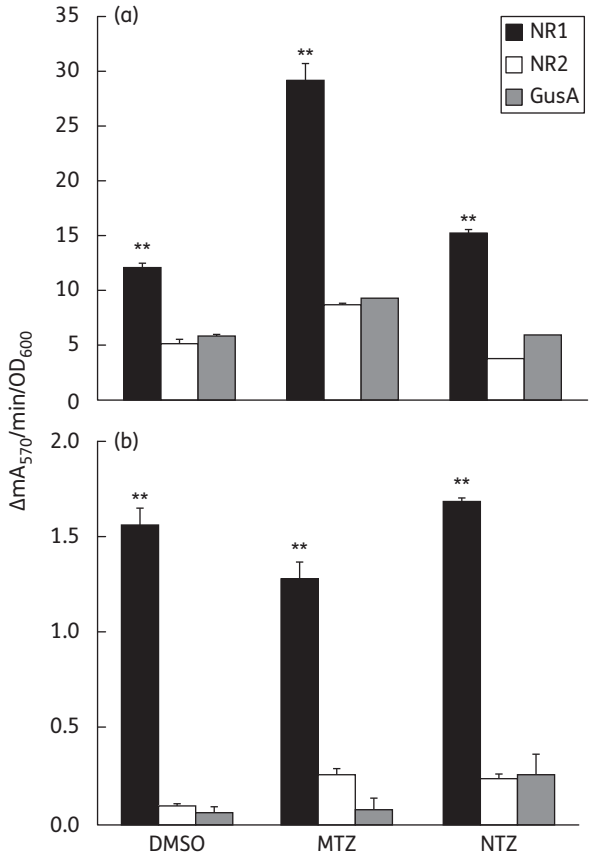

Figure 5. Responses of *E. coli* reporter strains to GlNR1 (black bars) and GlNR2 (white bars). GusA was included as a control (grey bars). *E. coli* RK4353/p*PnorV-lacZ*-1 (a) or SOS-R1 (b) strains transformed with the corresponding plasmids were grown in the presence of the nitro drugs metronidazole (MTZ) or nitazoxanide (NTZ), or DMSO as a solvent control until mid-log phase. β-Galactosidase activity was determined as described. Mean values ± SE are given for quadruplicates. Values marked by asterisks are significantly different from the control (paired *t*-test, two sided; **, $P<0.01$).

Taken together, these results suggest the following modes of action of both nitroreductases: (i) the essential ferredoxin domain transfers electrons from a donor (NADH) to the FMN in the active centre; (ii) in the case of GlNR1, the nitro compound is partially reduced, yielding toxic intermediates; and (iii) in the case of GlNR2, the nitro compound is entirely reduced yielding a non-toxic end product, e.g. the corresponding amine.

From an evolutionary point of view, the presence of functional and constitutively expressed nitroreductases in *G. lamblia* should represent at least one selective advantage. Otherwise, the respective sequences would be full of missense, or nonsense, mutations or have been deleted during evolution. These selective advantages could be: (i) inactivation of nitric oxide produced by endogenous processes (from L-arginine) or by the host; (ii) inactivation of nitroso or hydroxylamine intermediates present in the digestive tract; and (iii) utilization of naturally occurring nitrous compounds as alternative electron acceptors. These issues have been extensively discussed in the literature.[36]

In intestinal epithelial cells, the predominant nitric oxide biosynthetic pathway involves the inducible nitric oxide synthase converting free L-arginine into citrulline and nitric oxide.[37] Several publications suggest that nitric oxide produced in intestinal epithelium cells is a major defence molecule against *Giardia* infections. *In vitro*, nitric oxide inhibits the proliferation of trophozoites as well as cyst formation.[38] However, the effectiveness of nitric oxide against a giardial infection may be affected by the depletion of L-arginine as a consequence of a high-affinity uptake of the compound by the parasite[38] followed by degradation through two L-arginine-metabolizing enzymes (arginine deiminase and ornithine carbamoyl transferase) released by the parasite upon contact with host cells.[39,40] Under anaerobic conditions, nitroreductases may be candidates for the detoxification of endogenous, or exogenous, nitric oxide. Furthermore, nitroreductases may also be involved in the detoxification of nitroso compounds accumulating in the intestinal tract, particularly as a consequence of a red meat-enriched diet.[41] Such compounds may be generated at biological haem

centres mediating, e.g. the nitration of phenol and tryptophan.[42] Detoxification is, however, achieved only if the reduction of these compounds is complete. GlNR1 alone creates toxic intermediates; GlNR2 alone or together with GlNR1 would then achieve detoxification.

One should keep in mind, however, that the responses to nitric oxide and to nitro compounds may not be identical given the complexity of the associated chemical and biochemical reactions.[36] Differential transcriptomic and proteomic analyses[43] of suitable organisms (e.g. *E. coli*) expressing heterologous nitroreductases versus suitable controls may help in disclosing the roles of these enzymes.

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Transparency declarations

None to declare.

Supplementary data

Tables S1 and S2 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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