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Nitroreductases of bacterial origin in *Giardia lamblia*: Potential role in detoxification of xenobiotics

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Abstract

The anaerobic parasite *Giardia lamblia*, causative agent of persistent diarrhea, contains a family of nitroreductase genes most likely acquired by lateral transfer from anaerobic bacteria or archaebacteria. Two of these nitroreductases, containing a ferredoxin domain at their N-terminus, NR1, and NR2, have been characterized previously. Here, we present the characterization of a third member of this family, NR3. In functional assays, recombinant NR1 and NR3 reduced quinones like menadione and the antibiotic tetracycline, and—to much lesser extents—the nitro compound dinitrotoluene. Conversely, recombinant NR2 had no activity on tetracycline. *Escherichia coli* expressing NR3 were less susceptible to tetracycline, but more susceptible to the nitro compound metronidazole under semi-aerobic growth conditions. *G. lamblia* overexpressing NR1 and NR3, but not lines overexpressing NR2, are more susceptible to the nitro drug nitazoxanide. These findings suggest that NR3 is an active quinone reductase with a mode of action similar to NR1, but different from NR2. The biological function of this family of enzymes may reside in the use of xenobiotics as final electron acceptors. Thereby, these enzymes may provide at least two evolutionary advantages namely a higher potential to recycle NAD(P) as electron acceptors for the (fermentative) energy and intermediary metabolism, and the possibility to inactivate toxic xenobiotics produced by microorganisms living in concurrence inside the intestinal habitat.

KEYWORDS
anaerobic metabolism, detoxification, electron transfer, pathogenicity, resistance, susceptibility

1 | INTRODUCTION

Giardia lamblia is an anaerobic, but to some extent aerotolerant, unicellular eukaryote that causes persistent diarrhea in humans, domestic animals, and cattle (Cernikova, Faso, & Hehl, 2018). *G. lamblia* belongs to the phylum diplomonadids of the super-group Excavata (Adl et al., 2012). The entire genome has been sequenced (Morrison et al., 2007) and contains genes encoding for a variety of proteins involved in oxidative and nitrosative stress responses most likely acquired by lateral transfer (Ma'ayeh, Knorr, & Svard, 2015; Nixon et al., 2002). In previous studies, we have characterized two nitroreductases with N-terminal 4Fe-4S-ferredoxin domains followed by a NAD(P)H-FMN-nitroreductase domain, namely NR1 (Müller, Wastling, Sanderson, Müller, & Hemphill, 2007; Nillius, Müller, & Müller, 2011), annotated as Fd-NR2 (accession No. 22677) in GiardiaDB and NR2 (Müller, Schildknecht, & Müller, 2013), annotated

as Fd-NR1 (accession No. 6175) in GiardiaDB. For historical reasons—NR1 has been characterized first—we maintain our designations in the present study. In functional assays, both nitroreductases differ with respect to the action on nitrocompounds and have higher activities as quinone reductases than as nitroreductases (Müller et al., 2015). As published earlier, the closest homologues of NR1 and NR2 are oxygen-insensitive nitroreductases with N-terminal ferredoxin domains from the anaerobic deltaproteobacteria *Pelobacter carbinolicus* (NC_007498) and *P. propionicus* (NZ_AAJH01000004.1), and from the archaebacteria *Methanosarcina acetivorans* (NC_003552.1) and *M. barkeri* (NC_007355.1), all with probability scores of 2×10^{-25} or less. The *Giardia* genome features a gene encoding a NR family protein (accession No. 15307 in GiardiaDB). The corresponding polypeptide lacks a ferredoxin domain, but contains the nitroreductase domain homologous to the nitroreductase domains from NR1 and NR2. We henceforth refer to this polypeptide as NR3.

The primary structure of these NRs contains domains, that is ferredoxins (if present) and NAD(P) flavin reductase domains corresponding to the evolutionarily oldest polypeptides belonging to the "ancient anaerobic core" of around 60 protein families (Sousa, Nelson-Sathi, & Martin, 2016). The biological function is difficult to study in obligate anaerobes, especially in archaea, and therefore is still a question of debate (Martin & Sousa, 2016). In particular, it is unclear which evolutionary advantage their lateral transfer has conferred to eukaryotes such as *G. lamblia*. Here, we present a characterization of the third member of the NR family of *G. lamblia*, namely the polypeptide of NR3 overexpressed in *Escherichia coli* and in *G. lamblia*. In particular, this study investigates the quinone reductase activities of NR1, NR2, and NR3 on xenobiotics such as tetracycline.

2 | MATERIALS AND METHODS

2.1 | Biochemicals

If not otherwise stated, all biochemical reagents were from Sigma. Albendazole, dinitrotoluene, menadione, metronidazole, and nitazoxanide were kept as 100 mM stock solutions in DMSO at −20°C, and kanamycin and tetracycline (Figure 1) were kept as 2 mM stock solutions in DMSO at −20°C.

2.2 | Overexpression of recombinant NR in *E. coli*, purification, and functional assays

The open reading frame of NR3 was amplified from the *Giardia* genome using the primers NR3_F and NR3-R (see Appendix Table A1) obtaining a 532 base pair product as previously described for NR1 (Müller et al., 2007). For expression in BL21(DE3), cloning of NR1 (Müller et al., 2007; Nillius et al., 2011), NR2 (Müller et al., 2013), NR3 (this study), and GusA as a control (Nillius et al., 2011) in the *E. coli* His-tag expression vector system pET151 (pET151 directional TOPO; Invitrogen) was performed according to the instructions of the manufacturer. His-tag-purification of the recombinant NRs was performed as previously described for NR1 (Müller et al., 2007) and NR2 (Müller et al., 2013). Functional assays using the 0.2 µg of the recombinant nitroreductases were performed using the substrates (0.1 mM) as indicated, NADH (0.5 mM) as electron donor, and thiazolyl blue tetrazolium as a final electron acceptor as published (Müller et al., 2015).

2.3 | Determination of drug susceptibility in *E. coli*

Drug susceptibility of recombinant *E. coli* BL21(DE3) lines expressing either NR1, NR2, NR3, or GusA was tested by a conventional disk

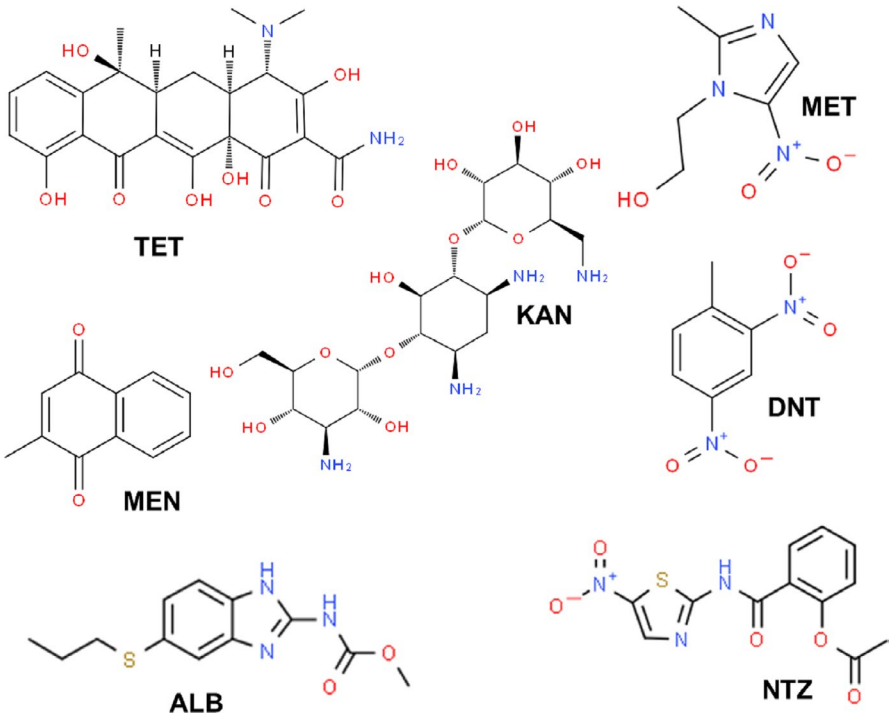

FIGURE 1 2-D-structure of compounds used in this study. ALB, albendazole; DNT, dinitrotoluene; KAN, kanamycin; MET, metronidazole; MEN, menadione; NTZ, nitazoxanide; TET, tetracycline

diffusion agar procedure as described previously (Müller et al., 2015, 2013). For this purpose, bacteria were grown to stationary phase (OD600 nm = 1) in Luria-Bertani medium (LB) containing 100 µg/ml ampicillin, and 0.3 ml of suspension was streaked on LB agar plates containing 100 µg/ml ampicillin. Whatman filter disks (5 mm diameter) were soaked with 7 µl of tetracycline, kanamycin (2 mM), or metronidazole (100 mM) stock solutions. The disks were air-dried for 5 min and 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 hr. Then, growth inhibition zone diameters were measured and the inhibition zone around the disk (see Appendix Figure 5) was calculated. For each compound, the values were expressed as a percentage of the mean value of the Gus control. Mean values ± *SE* are given for six replicates. Values marked by asterisks are significantly different to the Gus control.

2.4 | Determination of drug susceptibility in *G. lamblia*

The open reading frames of NR1, NR2, NR3, or GusA were amplified from the Giardia genome using the primers as listed in Table 3 and cloned into the vector pPacV-Integ (kindly provided by A. Hehl, Institute of Parasitology, Zürich, Switzerland) as described earlier (Müller et al., 2013; Nillius et al., 2011), with the sole exception that the strong promotor of the arginine deiminase gene (GL50803_112103) was used instead of the previously used GDH promotor (Leitsch, Müller, & Müller, 2016). Drug susceptibility of the resulting recombinant *G. lamblia* WBC6 lines was tested as described (Müller et al., 2013). Ninety-six-well-plates were inoculated with 10^3 trophozoites per well and grown in the presence of metronidazole, nitazoxanide, or albendazole at various concentrations. After 72 hr, growth of cells was monitored by a vitality assay based on the reduction in resazurin (Alamar Blue) to a pink product that was assayed by fluorimetry.

2.5 | Bioinformatics and statistical methods

Multiple sequence alignments were performed by protein–protein BLAST (Altschul et al., 1997) implemented at giardiadb.org. Alignments of GlNR3 and the nitroreductase domains of NR1 and NR2 were performed using lalign (embnet.vital-it.ch). The alignment presented in Figure 1 was produced by ClustalW, the phylogenetic tree by the T-Rex software package (both at trex.uqam.ca). The chemical structures were obtained from www.chemspider.com. The sequence of all recombinant constructs was verified by a custom sequencing service (Microsynth).

Statistical analysis of the inhibition tests was performed with suitable tools from the open-source software package R (R Core Team, 2012). The results were compared with the controls separately for each antibiotic and analyzed by two-sided paired *t* tests. Differences exhibiting values of $p < 0.005$ were considered significant (*), values of $p < 0.0001$ as highly significant (**). IC_{50} values were determined after logit-log-transformations of the data followed by regression analysis and are presented as mean values with 95% confidence intervals.

3 | RESULTS

Protein–protein Blast analysis of the 175 amino acid NR3 sequence predicted from the 525 bp ORF representing the entire gene revealed that the closest homologues were nitroreductases from anaerobic bacteria with amino acid identities of *c*. 30% and higher (Table 1). With the nitroreductase domains of NR1 and NR2, NR3 shared <20% identity, as shown in Table 1, and the detailed alignment in Figure 2a. A phylogenetic tree representing the three NRs relative to their closest homologues suggests that NR1 and NR2 have a common direct ancestor and that NR3 diverged earlier (Figure 2b).

After overexpression of NR3 in *E. coli* followed by His-tag affinity purification, a *c*. 24 kDa polypeptide was obtained corresponding to the *c*. 19 kDa from the NR3 sequence plus 5 kDa of the N-terminal leader sequence encoded by the vector (Figure 3a). In functional assays using NADH as an electron donor and menadione as an electron acceptor, the recombinant NR3 exhibited weaker activities than the recombinant full-length NR1 and NR2 (Figure 3b). With dinitrotoluene, all three recombinant enzymes had much lower activities than with menadione (Figure 3b and insert). Since the antibiotic tetracycline is a quinone (see Figure 2), we tested whether tetracycline could also be

TABLE 1 List of the five closest non-giardial homologues to NR3 (Giardia DB 15307)

Organism	Accession	E value	Identity (%)
Fusobacterium sp.	WP_101474820.1	2×10^{-23}	33.8
Thermovirga lienii	WP_014163980.1	4×10^{-21}	34.4
Desulfomicrobium baculatum	WP_012805745.1	3×10^{-19}	33.8
Clostridiisalibacter paucivorans	WP_012805745.1	10^{-19}	30.6
Dethiosulfatibacter aminovorans	WP_073046065.1	3×10^{-18}	29.9
Giardia lamblia NR1	Giardia DB 22677	5×10^{-9}	19.7
G. lamblia NR2	Giardia DB 6175	10^{-6}	19.4

Note: All were annotated as nitroreductases. *E* values and amino acid identities for the giardial NR1 and NR2 have been included.

(a)

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NR1  MVEGYPVFLTDTCTGCNMCVEVCPTAVLKVDPETKVVAYANRDNCIFCGHCGAICPTESI 60
NR2  -MSRFPEVDEDLCIGCNVCVQGCPTQCIEVNAETRKLVFRAKETCISCGHCASVCRTAAI 59
NR3  ------------------------------------------------------------

NR1  SMFGITPESEENAIKTDPPAVRAVKMARTCRKYHPEPLPKEDLMQALSIAKYSASSSNIR 120
NR2  SMFGILPDTVEEEIMADKPVVRSIKMARQHRFFRSTSISPQEVLRILNIAKYSGANAYNS 119
NR3  ----------------MPPICDAILRRRAIKQYTKESVSIEAIEYLRKVAVAIPTGHNTR 44
                      *   ::   *  : :   .:. : :    .:*    :.

NR1  PLHFTVLS--RSLMDTLSLSIAKEVSKNPRYSKVAALVEKGIDVVFRGAPHMILMSA--- 175
NR2  AFHFTAVDLKRNNVDAVIEVFVTALSKTSKYAWLPKVRAHGGDPIFHGAPWLILISTGHN 179
NR3  HTEFAFVTN-----PRIIKTISQAKGEKAEYMQHAKLLIVVMGIQNDGVTSIATDMS--- 96
       .*: :         :   :    .:...*   . :     .    *.. :    :

NR1  ---PADKAA--VAMADASIAGRDIQLNAESMGLGMFWCGFLLAAVASSQELHDICG---- 226
NR2  EDDPKDDGPGLDAFTQAHMLYRDLQLHSEIRGYGTHLCRILQPHLNDVQSLSDSCPNIFD 239
NR3  ----IAAAMIQLACSDFGLACSWEQFYGRKNVYGEDSERIVLDVLRLLN---------- 141
           .    * ::  :     *: ..    *     ::   :   :

NR1  VPEGHKILMAMGIGRPKVKFARPALRRNLEEGVDITFK- 264
NR2  VPQSHTVLLAFVLGIGAMKYHRPLLLPDLVEGIDVTFVE 278
NR3  -SPNRRVLCALAIGYPAVQMPP----ADMHENGRIYMIE 175
      . .: :* *: :*   ::        :: *.  : :
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4 Fe-4S-Ferredoxin-domain

Nitroreductase-domain

(b)

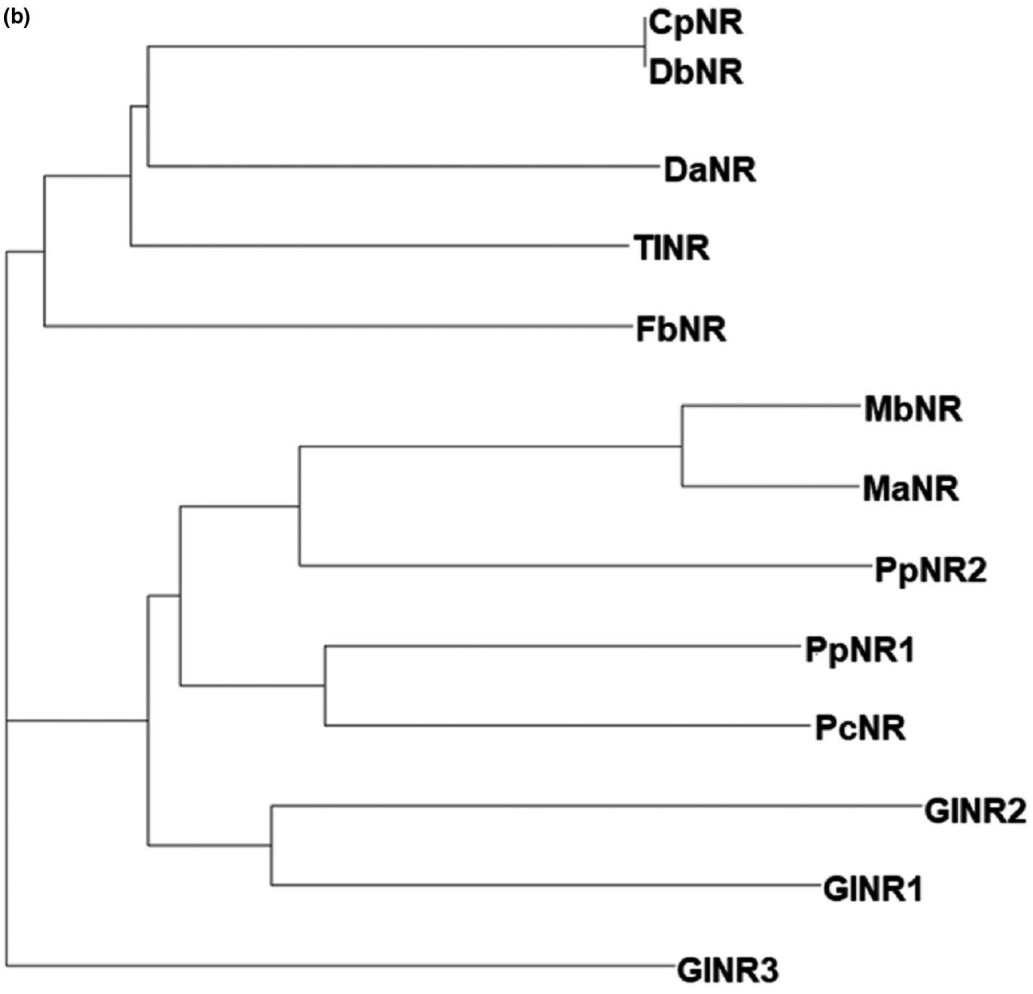

FIGURE 2 (a) Alignment of three nitroreductases from *Giardia lamblia* WBC6, namely Fd-NR1 (Giardia DB 6,177; referred to as NR2), Fd-NR2 (Giardia DB 22,677; referred to as NR1), and the NR family protein (Giardia DB 15,307; referred to as NR3). Symbols below the sequence alignment indicate positions with amino acids shared (*) or similar chemical groups (:) between all three proteins, or shared or positively related between two proteins only (.). (b) Phylogenetic tree presenting the *G. lamblia* NRs relative to their closest homologues. CpNR, *Clostridiisalibacter paucivorans* WP_012805745.1; DaNR, *Dethiosulfatibacter aminovorans* WP_073046065.1; DbNR, *Desulfomicrobium baculatum* WP_012805745.1; FbNR, *Fusobacterium sp.* WP_101474820.1; MaNR, *Methanosarcina acetivorans* AAM06120.1; MbNR, *Methanosarcina barkeri* AAZ72168.1; PcNR, *Pelobacter carbinolicus* WP_011340188.1; PpNR1, *Pelobacter propionicus* WP_011735747.1; PpNR2, *P. propionicus* WP_011734719.1; TlNR, *Thermovirga lienii* WP_014163980.1 (all annotated as nitroreductases)

reduced by the recombinant NRs. Interestingly, only the recombinant NR1 and NR3, but not NR2, could use tetracycline as electron acceptor. Both enzymes preferred it as compared to thiazolyl blue tetrazolium (Figure 3b including insert).

These findings prompted us to investigate whether *E. coli* expressing recombinant NRs were less susceptible to tetracycline than a control strain overexpressing an irrelevant enzyme, namely glucuronidase (GusA) from *E. coli*. Since *E. coli* expressing recombinant NR1 and NR2 showed a different phenotype when exposed to metronidazole NR1 increasing, NR2 abolishing the susceptibility (Müller et al., 2015) as compared to the GusA control strain under semi-aerobic conditions, we included both strains in our experiment. Interestingly, semi-aerobically grown *E. coli* expressing NR1 and NR3, but not NR2 had significantly higher resistance to tetracycline than the GusA strain control. In the presence of metronidazole, the NR1 and NR2 strains exhibited the same phenotype as previously shown, NR1 increasing and NR2 significantly decreasing the susceptibility. Surprisingly, the strain expressing NR3 had a significantly higher susceptibility to metronidazole than the Gus strain. Susceptibility to the non-quinone antibiotic kanamycin was not significantly affected in any of the tested strains (Figure 4). Under aerobic conditions, the susceptibilities to TET were not affected (Appendix Figure 6).

When overexpressed in *G. lamblia* under ADI-promotor control, NR1 and NR3, but not NR2, increased the susceptibility to nitazoxanide significantly as compared to a strain overexpressing GusA (non-overlapping 95% intervals). In all three strains, susceptibility to metronidazole was only slightly affected (lower mean values,

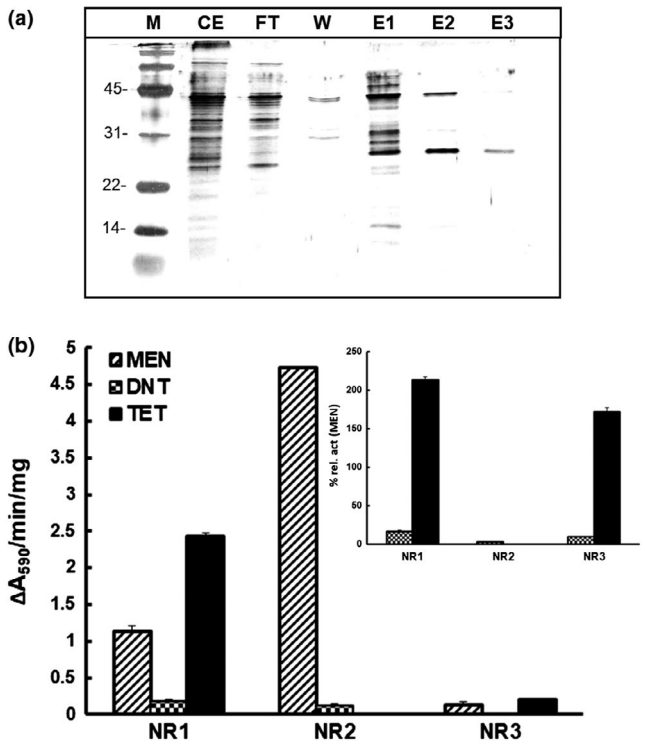

FIGURE 3 Heterologous expression in *Escherichia coli* Bl21(DE3) and functional assay of NR3. (a) SDS-PAGE of His-tag-purified recNR3. M, markers with sizes in kDa, CE, crude extract, FT, flow-through, W, wash with 10 mM imidazole, E1–E3, fractions eluted with 200 mM imidazole. (b) functional assay of fraction E3 in the presence of menadione (MEN), dinitrotoluene (DNT), or tetracycline (TET). Functional assays were performed with NADH as an electron donor and thiazolyl blue tetrazolium (MTT) as chromogenic electron acceptor. The reaction was performed at 37°C under normal atmosphere and stopped after 2 hr by addition of one volume pure ethanol. Mean values (±*SE*) are given for three replicates. The insert represents the activities on DNT or TET as percentage of the activities on MEN

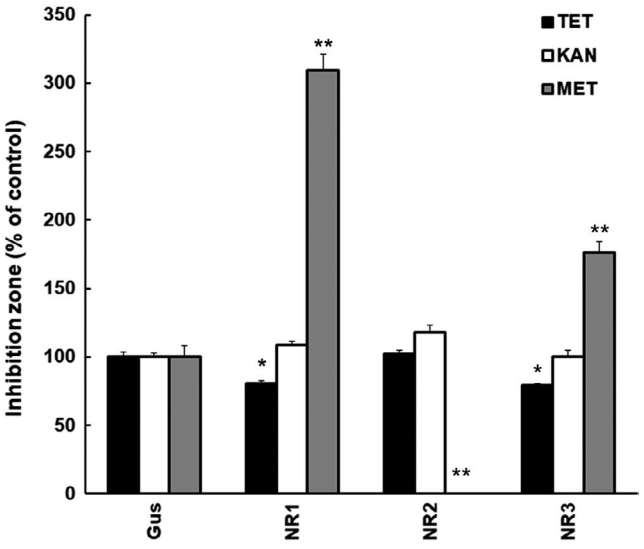

FIGURE 4 Susceptibility of *Escherichia coli* BL21(DE3) expressing GusA as a control (Gus), GlNR1 (NR1), GlNR2 (NR2), or GlNR3 (NR3) to tetracycline (TET), metronidazole (MET), or kanamycin (KAN). Lines were plated, disks containing the drugs were added, and plates were incubated under semi-aerobic conditions. After 24 hr, diameters of inhibition zones were determined, the surfaces of inhibition zones were calculated, and the values were processed as described in Materials and Methods. The results were compared with the controls separately for each antibiotic and analyzed by two-sided paired *t* tests. Differences exhibiting values of $p < 0.005$ were considered significant (*), values of $p < 0.0001$ as highly significant (**).

TABLE 2 Susceptibility of *Giardia lamblia* WBC6 expressing *Escherichia coli* beta-glucuronidase A (GusA) or *G. lamblia* nitroreductase homologues (NR1, NR2, and NR3) to metronidazole (MET), nitazoxanide (NTZ), or albendazole (ALB)

Strain	MET (μM)	NTZ (nM)	ALB (nM)
GusA	1.2 (0.6–1.8)	32 (16–48)	19 (15–21)
NR1	0.5 (0.3–0.7)	**0.2 (0.1–0.3)**	18 (15–21)
NR2	0.7 (0.5–0.9)	27 (17–37)	19 (13–25)
NR3	0.8 (0.6–1.0)	**3.4 (2.1–4.7)**	20 (15–25)

Note: Mean IC_{50} values and 95% confidence intervals are given. Values significantly differeing from GusA are in bold.

but overlapping intervals). Susceptibility to albendazole was not affected in any strain (Table 2).

4 | DISCUSSION

As for NR1 and NR2, the closest homologues of NR3 are nitroreductases from anaerobic bacteria. Therefore, we can postulate that the gene encoding for NR3 was also acquired by an ancestor of *G. lamblia* through lateral transfer from an (unknown) anaerobic bacterium or archaebacterium. In functional assays, similar to the previously characterized NR1 and NR2, the recombinant NR3 has a higher quinone reductase than nitroreductase activity, thereby confirming that this enzyme family is multifunctional, using quinones as well as nitro compounds as electron acceptors. NR1 and NR3 can use the quinone antibiotic tetracycline as a substrate. Expression of both enzymes in *E. coli* confers some protection from tetracycline suggesting that the resulting product, likely a semiquinone, may be less cytotoxic. Unlike the in vivo reduction in metronidazole, this works only under semi-aerobic conditions suggesting that the presence of oxygen under atmospheric pressure inhibits the reduction in tetracycline or re-oxidizes the product. In contrast to these in vivo assays, the functional assays are operational under aerobic laboratory conditions since the electrons are transferred from the reduced substrate to thiazolyl blue tetrazolium as a final (and stable) acceptor. Interestingly, although in functional assays, activity on metronidazole is marginal as compared to other substrates in the case of NR1 and NR2 (Müller et al., 2015) or non-existent in the case of NR3 (this study), *E. coli* expressing these three enzymes exhibit distinct phenotypes exposed to metronidazole. This could be explained by the fact that metronidazole has a very low midpoint redox potential. Therefore, the nitroreductases need other cofactors or interaction partners such as free ferredoxins with a similarly low potential that are not present in the in vitro assay (Leitsch, 2017b). A fusion construct of NR3 and the ferredoxin domain of NR2 at its N-terminus (see Appendix for details) expressed in *E. coli* does not confer increased susceptibility to metronidazole or increased resistance to tetracycline as compared to the original NR3 (Appendix Figure 7). Another possibility is that metronidazole is reduced in *E. coli* by endogenous nitroreductases independently of the presence of *Giardia* NRs. The reduced intermediate reacts with other micrometabolites such as nucleotides (Ludlum, Colinas, Kirk, & Mehta, 1988) or amino acids (Leitsch, Kolarich, Wilson, Altmann, & Duchene, 2007), thereby generating the electron acceptors for the *Giardia* NRs which in turn enhance (NR1 and NR3) or reduce (NR2) their toxicities.

Results obtained with *G. lamblia* overexpressing the NRs show a different picture and are different from previously published findings on NR1 (Nillius et al., 2011) and NR2 (Müller et al., 2013). In the present study, NR1 and NR3 overexpressors are more susceptible to the nitro drug nitazoxanide, but not to metronidazole, and NR2 overexpressors are not significantly different from the wild type. Although considered as a "standard method," gain-of-function studies via overexpression of selected genes in *Giardia* are difficult. As a response to the puromycin selection of transformed trophozoites, the strain WBC6 responds by an inherent instability of gene expression ("antigenic switch") as evidenced in earlier work (Su, Lee, Huang, Chen, & Sun, 2007) and by own, unpublished studies. Therefore, comparisons between transformed lines issuing from different batches are intrinsically different, apart from different external conditions such as the culture medium and the overexpression constructs (GDH promotor in previous studies vs. the stronger ADI promotor in the present study). Moreover, the susceptibilities to the nitro drugs metronidazole and nitazoxanide, but not to albendazole, strongly depend on the medium composition, not only the cysteine content (Leitsch, 2017a), but also batches of serum and peptone and therefore cannot be directly compared between studies ranging over a decade. This becomes visible if the IC_{50} values of control strain GusA are compared between our previous studies and the present one (See Appendix Table A2). Moreover, we have noticed a strong increase in the susceptibility to nitazoxanide (in the GusA control as well as in untransformed wild-type trophozoites) on media prepared with novel batches of serum and peptone. This may explain why exposure to nitazoxanide gives a much better read-out with respect to increased nitro drug susceptibility in NR1. NR3 resembles much more to NR1 than to NR3 both strongly increasing the susceptibility to nitazoxanide thereby confirming the *E. coli* overexpression assays which are highly reproducible—even between different studies.

Taken together, these results suggest that the ancestor of *G. lamblia* has acquired genes encoding for the NR homologues from anaerobic bacteria, thereby enhancing its capability to use xenobiotics as electron acceptors. This might confer a selective advantage via a higher potential to recycle NAD(P) as electron acceptors for the (fermentative) energy and intermediary metabolism, or via the possibility to inactivate toxic xenobiotics produced by gut commensal microorganisms. Recently, similar findings were published concerning the *Haemophilus influenzae* nitroreductase NfsB reducing and thereby inactivating chloramphenicol (Crofts et al., 2019). On the other hand, the reduction in nitro compounds to toxic intermediates renders organisms expressing such nitroreductases susceptible to nitro drugs and therefore constitutes a selective disadvantage. In the case of *G. lamblia*, nitro drug resistance is, however, frequent and in some cases linked to the

downregulation of nitroreductases (Emery et al., 2018; Müller, Braga, Heller, & Müller, 2019).

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CONFLICT OF INTERESTS

None declared.

AUTHOR CONTRIBUTIONS

Both authors: conceptualized, formally analyzed, and wrote the manuscript. N. Müller: involved in funding acquisition.

ETHICS STATEMENT

None required.

DATA AVAILABILITY STATEMENT

All data generated or analyzed during this study are included in this published article and in the Appendix.

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APPENDIX

CONSTRUCTION AND TESTING OF GLNR3 FUSED TO THE FERREDOXIN DOMAIN OF GLNR2

METHODS

In order to test whether the activities of GlNR3 (GL50803_15307) are enhanced by adding a ferredoxin (Fd)-domain, we have generated a chimeric construct with the Fd-domain of GlNR2 (GL50803_6175) at its N-terminus and the full-length GlNR3 at its C-terminus. We have amplified the coding sequence of GlNR3 using the primers NR3_fusion_F and NR3_fusion_R and cut the resulting product with the restriction enzymes HincII and SacI. The resulting fragment was cloned into a PET-His151 vector containing the full-length GlNR2 (Müller et al., 2013) cut with the same enzymes thereby removing the nitroreductase domain of GlNR2. The resulting clones were sequenced and a construct with the correct sequence—henceforth referred to as Fd-NR2-NR3—was transformed into *Escherichia coli* Bl21. The resulting strain was compared with strains expressing GlNR3 or GusA as a control in disk diffusion tests with tetracycline (TET) or metronidazole (MET) as inhibitors.

RESULTS

As shown in Figure 7, transformation with the fusion construct Fd-NR2-NR3 did not increase the susceptibility to MET nor decrease susceptibility to TET as compared to NR3 only. *E. coli* expressing GlNR3 was significantly more susceptible to MET ($p < 0.001$) and less susceptible to TET ($p < 0.005$) thereby confirming the results shown in Figure 4.

TABLE A2 Comparison of drug susceptibilities of the control strain GusA between different publications referred to as Publication 1 (Nillius et al., 2011), publication 2 (Müller et al., 2013), and the present publication. The concentrations and the 95% confidence intervals are given in µM

Drug	Publication 1	Publication 2	Present publication
Albendazole	0.04 (0.01)	0.03 (0.01)	0.02 (0.002)
Metronidazole	4.5 (0.9)	1.7 (0.3)	1.2 (0.6)
Nitazoxanide	1.5 (0.3)	0.7 (0.1)	0.03 (0.015)

TABLE A1 Overview of primers used in this study (utr., untranslated). Please note that the nitroreductase GlNR1 is annotated as Fd-NR2, that GlNR2 is annotated as Fd-NR1, and that GlNR3 is annotated as nitroreductase family protein in the *Giardia* data base (http://giardiadb.org/giardiadb/). The coding sequences are underlined. ADI, arginine deiminase (GL50803_112103)

Name	Sequence	Gene (accession number)	Use
NR3_F	CACCATGGTTGAAGGTTATCCTG	Nitroreductase family protein GlNR3 (GL50803_15307)	Cloning into pET-His151 for expression in *Escherichia coli*
NR3_R	TTACTCTATCATATAAATTCGTC		Idem
NR3_fusion_F	GAGTCGACCCTCCCATTTGTGACGCT		Introduction of a HincII-site at the 5'-end of NR3.
NR3_fusion_R	GAGAGCTCTTACTCTATCATATAAATTCGTC		Introduction of a SacI-site at the 3'-end of NR3, both for cloning into pET-His151 containing the GlNR2 coding sequence.
GlNR1_ADI_F	GATCTAGAAACGTCTACACGTGAGGTG TGTAAACTTCCGGAGAAAAAAAT CCTAGTACATGGTTGAAGGTTATCCTG	Fd-NR2 GlNR1 (GL50803_22677)	Introduction of a XbaI-site and of the ADI promotor at the 5'-end of NR1.
GlNR1_ADI_R	GATTAATTAACTGGATATGAACATGTCAAT TATTTGATATCTGAATTACAATTCACTGT TTTACTTAAATGTAATGTCGAC		Introduction of a PacI-site and of the ADI-downstream region at the 3'-end of NR1.
GlNR2_ADI_F	GAGCTAGCAACGTCTACACGTGAGGTG TGTAAACTTCCGGAGAAAAAAATCC TAGTACATGTCACGCTTTCCAGAGG	Fd-NR1 GlNR2 (GL50803_6175)	Introduction of a NheI-site and of the ADI promotor at the 5'-end of NR2.
GlNR2_ADI_R	GATTAATTAACTGGATATGAACATGTCA ATTATTTGATATCTGAATTACAATTCAC TGTTTTATTCCACAAACGTTACGTCTA TCTTAAATGTAATGTCGAC		Introduction of a PacI-site and of the ADI-downstream region at the 3'-end of NR2.
GlNR3_ADI_F	GATCTAGAAACGTCTACACGTGAGGT GTGTAAACTTCCGGAGAAAAAAAT CCTAGTACATGCCTCCCATTTGTGAC		Introduction of a XbaI-site and of the ADI promotor at the 5'-end of NR3.
GlNR2_ADI_R	GATTAATTAACTGGATATGAACATGTC AATTATTTGATATCTGAATTACAATT CACTGTTTTATTCCACAAACGTTAC GTCTATCTTAAATGTAATGTCGAC		Introduction of a PacI-site and of the ADI-downstream region at the 3'-end of NR3.

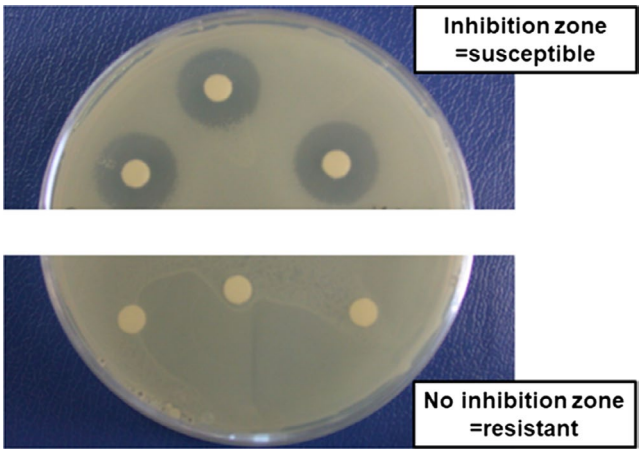

FIGURE A1 Typical disk diffusion assay. The disks contained either kanamycin (2 mM; upper panel) or DMSO as a solvent control (lower panel)

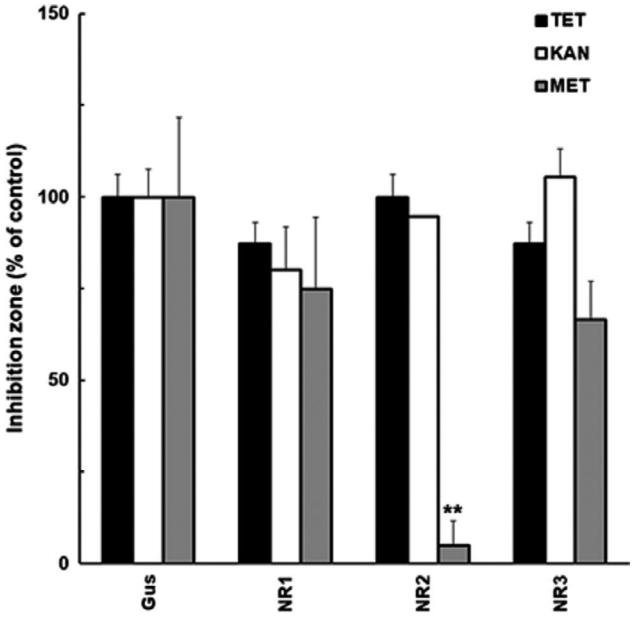

FIGURE A2 Susceptibility of *Escherichia coli* BL21(DE3) expressing GusA as a control (Gus), GlNR1 (NR1), GlNR2 (NR2), or GlNR3 (NR3) to tetracycline (TET), kanamycin (KAN), or metronidazole (MET). The tests were performed under aerobic conditions as described in Materials and Methods. Mean values ± *SD* are given for three replicates

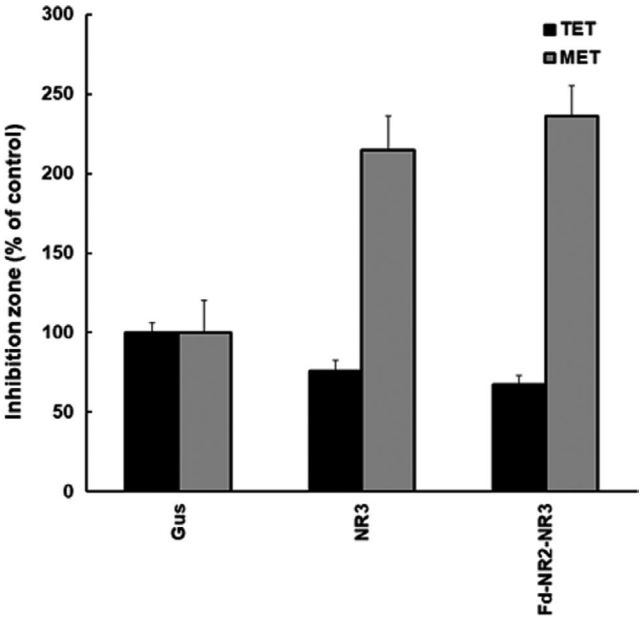

FIGURE A3 Susceptibility of *Escherichia coli* BL21(DE3) expressing GusA as a control (Gus), GlNR3 (NR3), or the fusion of the ferredoxin domain of GlNR2 and GlNR3 (Fd-NR2-NR3) to tetracycline (TET), or metronidazole (MET). The tests were performed under semi-aerobic conditions as described in Materials and Methods. Mean values ± *SD* are given for four replicates