**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 from anaerobic bacteria or archaeabacteria. 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
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 $2 \times 10^{-25}$ 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 archaebacteria, 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](https://via.placeholder.com/150) 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% O2, 10% CO2, 85% N2) 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 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 |
| *Thermotoga* | WP_014163980.1 | 4 × 10^-21 | 34.4 |
| *Desulfomicrobium* baculatum | WP_012805745.1 | 3 × 10^-19 | 33.8 |
| *Clostridislabilbacter* paucivorans | WP_012805745.1 | 10^-19 | 30.6 |
| *Dethiosulfatabacter* aminovorans | WP_073046065.1 | 3 × 10^-18 | 29.9 |
| *G. 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.
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,
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.

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).

ACKNOWLEDGMENTS
This work was supported by the Swiss National Science Foundation (grant No. 31003A_163230).

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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How to cite this article: Müller J, Müller N. Nitroreductases of bacterial origin in Giardia lamblia: Potential role in detoxification of xenobiotics. MicrobiologyOpen. 2019;8:e904. https://doi.org/10.1002/mbo3.904
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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 BI21. 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 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://giardiaadb.org/giardiaadb/). The coding sequences are underlined. ADI, arginine deiminase (GL50803_112103)

| Name            | Sequence                              | Gene (accession number) | Use                                                                 |
|-----------------|---------------------------------------|-------------------------|----------------------------------------------------------------------|
| NR3_F           | CACCATGGTTGAAGGGTTATCTTG              | Nitroreductase family protein GlNR3 (GL50803_15307) | Cloning into pET-His151 for expression in Escherichia coli          |
| NR3_R           | TTACTCTACATAAATCGTC                    |                         |                                                                    |
| NR3_fusion_F    | GAGTCGACCTCCCCAGTTTGACGCT             |                         | Idem                                                                |
| NR3_fusion_R    | GAGAGCTTTACATCTATATATATCCGT           |                         | Introduction of a SacI-site at the 3'-end of NR3.                  |
| GlNR1_ADI_F     | GATCTAGAACCGCTACACGGAAGGTTG           | Fd-NR2 GlNR1 (GL50803_22677) | Introduction of a XbaI-site and of the ADI promoter at the 5'-end of NR1. |
| GlNR1_ADI_R     | GATTAATTAACTGGATATGAGAACTTGCAAT       |                         | Introduction of a PacI-site and of the ADI-promoter at the 3'-end of NR1. |
| GlNR2_ADI_F     | GAGCTAGCAACGGTCTACACTGGAGGT           | Fd-NR1 GlNR2 (GL50803_6175) | Introduction of a XbaI-site and of the ADI promoter at the 5'-end of NR2. |
| GlNR2_ADI_R     | GATTAATTAACCTGATATGGAAACTTGCA         |                         | Introduction of a PacI-site and of the ADI-downstream region at the 3'-end of NR2. |
| GlNR3_ADI_F     | GATCTAGAACGGTCTACACGGAAGGTTG          |                         | Introduction of a XbaI-site and of the ADI promoter at the 5'-end of NR3. |
| GlNR2_ADI_R     | GATTAATTAACCTGATATGGAAACTTGCA         |                         | Introduction of a PacI-site and of the ADI-downstream region at the 3'-end of NR3. |

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)        |
**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), GINR1 (NR1), GINR2 (NR2), or GINR3 (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), GINR3 (NR3), or the fusion of the ferredoxin domain of GINR2 and GINR3 (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.