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Assessing the Essentiality of *Leishmania donovani* Nitroreductase and Its Role in Nitro Drug Activation

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The nitroimidazole fexinidazole has potential as a safe and effective oral drug therapy for the treatment of visceral leishmaniasis. To date, nitroheterocyclics have not been used in the treatment of leishmaniasis, and relatively little is known about their mechanism of action. In African trypanosomes, nitro drugs are reductively activated by a type I nitroreductase (NTR), absent in mammalian cells. Modulation of nitroreductase levels in *Trypanosoma brucei* directly affected sensitivity to nitro compounds, with reduced concentrations of the enzyme leading to moderate nitro drug resistance. In view of the progression of fexinidazole into clinical development for visceral leishmaniasis, here we assess the essentiality of the nitroreductase in *Leishmania donovani* and the effect of modulating nitroreductase levels on susceptibility to fexinidazole. The failure to directly replace both endogenous copies of the *NTR* gene, except in the presence of an ectopic copy of the gene, suggests that the *NTR* gene is essential for the growth and survival of *L. donovani* promastigotes. Loss of a single chromosomal copy of the *L. donovani NTR* gene resulted in parasites that were mildly resistant (<2-fold) to the predominant *in vivo* metabolite of fexinidazole, while parasites overexpressing NTR were 18-fold more susceptible. These data confirm that *Leishmania* NTR plays a pivotal role in fexinidazole activation. Reliance on a single enzyme for prodrug activation may leave fexinidazole vulnerable to the emergence of drug resistance. However, the essentiality of the NTR in *L. donovani* promastigotes, combined with the limited resistance shown by *NTR* single knockout cells, suggests that the potential for the spread of NTR-based resistance to fexinidazole may be limited.

Visceral leishmaniasis (VL), caused by the protozoan parasite *Leishmania donovani*, is the second largest parasitic killer after malaria, with more than 200 million people currently at risk from infection. In 95% of cases, death can be prevented by timely and appropriate drug therapy (1); however, current treatment options are far from ideal. In India, Bangladesh, and Nepal, the epicenter of VL infection, with 60% of the world's reported cases (2), concerted efforts are being made to eradicate the disease by 2015. At present, the best available treatments for anthroponotic VL are miltefosine and liposomal amphotericin B. Undoubtedly, both drugs are vastly superior to previous therapies, but they also have limitations. The principal drawbacks of miltefosine are its teratogenicity, prolonged treatment regimen, and high resistance potential (3). Problems associated with amphotericin B include high treatment costs, an intravenous route of administration, and unresponsiveness in some Sudanese VL patients (4). Thus, there remains a definite and urgent need to strengthen the range of treatment options for VL.

In the search for more-effective treatments for neglected diseases, there has been renewed interest in the chemotherapeutic potential of nitroheterocyclic compounds. This interest stems largely from the success of nifurtimox-eflornithine combination therapy (NECT) for the treatment of the Gambian form of human African trypanosomiasis (HAT) (5). Treatment with NECT, consisting of oral nifurtimox over 10 days with eflornithine infusions for 7 days, has resulted in cure rates of ca. 97%, leading to its inclusion on the Essential Medicines List of the WHO (5). The success of nifurtimox as part of NECT prompted the Drugs for Neglected Diseases Initiative (DNDi) to undertake a comprehensive screen of 700 nitroheterocyclic compounds for antiparasitic activity. As a result, the 2-substituted 5-nitroimidazole fexinidazole (Hoe 239), first shown to have antitrypanosomal activity almost 30 years ago (6), was rediscovered. Subsequently, phase I clinical trials for treating African sleeping sickness with fexinidazole have been successfully completed, and phase II/III trials are scheduled for later this year (7). Fexinidazole also has potential as a safe and effective oral drug therapy for the treatment of visceral leishmaniasis (8). Administration of a once-daily oral regimen of 200 mg of fexinidazole kg of body weight^{-1} over 5 days was found to suppress infection in a mouse model of visceral leishmaniasis by 98.4%, a potency comparable to that seen with the current frontline antileishmanial miltefosine. In light of these findings, fexinidazole is now scheduled to enter phase II clinical trials for the treatment of visceral leishmaniasis in Sudan (Bernard Pécoul, DNDi, personal communication).

Nitroheterocyclics have not been used in the treatment of leishmaniasis, and as a result, relatively little is known about their mechanism of action against leishmania parasites. In African trypanosomes, the mode of action of nifurtimox involves reductive activation via a type I NADH-dependent nitroreductase (NTR), resulting in the generation of a cytotoxic, unsaturated open-chain nitrile derivative (9). Overexpression of the leishmanial homolog of this nitroreductase in *L. donovani* was found to increase sensitivity to fexinidazole 15-fold and sensitivity to nifurtimox 19-fold (8), indicating that similar mechanisms may be involved in *L. donovani* and the African trypanosome, *Trypanosoma brucei*. Genetic studies indicate that nitroreductase is essential for the survival of the African trypanosome *in vitro* (10). However, modula-

Received 29 August 2012 **Returned for modification** 11 October 2012
Accepted 26 November 2012
Published ahead of print 3 December 2012
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doi:10.1128/AAC.01788-12
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tion of the nitroreductase levels within trypanosomatids has been shown to directly affect sensitivity to nitro compounds *in vitro*, with reduced enzyme activity leading to moderate nitro drug resistance (10). In view of the progression of fexinidazole into clinical development for visceral leishmaniasis, it is clear that the potential for nitroreductase-mediated drug resistance should also be addressed for *Leishmania* spp. With this in mind, here we assess the essentiality of the nitroreductase in *Leishmania donovani* and the effect of the modulation of nitroreductase levels on susceptibility to nitro drugs.

MATERIALS AND METHODS

Ethics statement. All animal experiments were approved by the Ethical Review Committee at the University of Dundee and were performed under the Animals (Scientific Procedures) Act 1986 (United Kingdom Home Office Project License PPL 60/4039) in accordance with the European Communities Council Directive (86/609/EEC).

Cell lines and culture conditions. The clonal *Leishmania donovani* cell line LdBOB (derived from MHOM/SD/62/1S-CL2D) was grown as promastigotes at 26°C in modified M199 medium, as described previously (11).

Test compounds. Fexinidazole sulfone was synthesized as described previously (8). Compound purity was determined by liquid chromatography–mass spectrometry (LC-MS) and was found to be ≥95%. The stocks of nifurtimox used in this study were provided by Bayer, Argentina, as a kind gift. Nitrofurazone and miltefosine were purchased from Sigma-Aldrich Ltd.

***In vitro* drug sensitivity assays.** To examine the effects of test compounds on growth, triplicate promastigote cultures were seeded with 1×10^5 parasites ml^{-1}. Parasites were grown in 5-ml cultures in the presence of a drug for 72 h, after which 200-μl aliquots of each culture were added to 96-well plates; 50 μM resazurin was added to each well; and fluorescence (excitation wavelength, 528 nm; emission wavelength, 590 nm) was measured after a further 4 h of incubation (12). Data were processed using GRAFIT (version 5.0.4; Erithacus Software) and were fitted to a 2-parameter equation, where the data are corrected for background fluorescence, to obtain the effective concentration inhibiting growth by 50% (EC_{50}):

$$y = \frac{100}{1 + \left(\frac{[I]}{EC_{50}}\right)^m}$$

In this equation, $[I]$ represents the inhibitor concentration and m is the slope factor. The experiments were repeated at least three times, and the data are presented as the weighted means plus weighted standard deviations (12).

Amastigote drug sensitivity assays. In-macrophage drug sensitivity assays were carried out as described previously (8), using starch-elicited peritoneal macrophages harvested from BALB/c mice. Macrophages were infected with metacyclic promastigotes at a ratio of 10 promastigotes to 1 macrophage.

Generation of knockout, overexpression, and recovery constructs. The primers used to generate constructs for genetic manipulation were designed using the *Leishmania infantum* nitroreductase (*NTR*) sequence in GeneDB (LinJ.05.0660) as a template (Table 1). The accuracy of all constructs assembled was verified by sequencing. *NTR* gene replacement cassettes were generated by amplifying a region of DNA encompassing the 5′ untranslated region (5′ UTR), open reading frame (ORF), and 3′ UTR of LdBOB *NTR* from genomic DNA with primers 5′UTR-NotI_s and 3′UTR-NotI_as, using *Pfu* polymerase. This sequence was then used as a template for the amplification of the individual regions used in the assembly of replacement cassettes containing the selectable drug resistance genes encoding puromycin *N*-acetyltransferase (*PAC*) and hygromycin phosphotransferase (*HYG*), constructed exactly as described previously (13).

TABLE 1 Cloning primers

Primer name	Primer sequence[a]
5′UTR-NotI_s	5′-ataagaatgcggccgcAGTCATGTGATGGTG GTTTACGGCA-3′
5′UTR-HindIII/PmeI_as	5′-gtttaaacttacggaccgtcaagcttTGTAGTTTCT CTTGCTGCTTTCC-3′
3′UTR-PmeI/BamHI_s	5′-gacggtccgtaagtttaaacggatccTTTAGACTCG GCACCAGAGATGAGA-3′
3′UTR-NotI_as	5′-ataagtaagcggccgcCCAAGACAGCAGCA GCCGGTCGTGA-3′
LdNTR-BamHI_s	5′-ggatccATGCTTCGCCGCAGTCGCCGCTT-3′
LdNTR-BamHI_as	5′-ggatccTAGAACTTGTTCCACCGCACGGT-3′

[a] Capital letters represent nucleotides corresponding to gene sequences in *L. infantum*; lowercase letters represent additional sequences used in generating constructs. Restriction endonuclease sites are underlined.

The recovery vector was generated by amplifying the LdBOB nitroreductase gene from genomic DNA using the *LdNTR*-BamHI sense and antisense primers. The PCR product was then cloned into the pCR-Blunt II-TOPO vector (Invitrogen) and was sequenced. The pCR-Blunt II-TOPO-*Ld*NTR construct was then digested with BamHI, and the fragment was cloned into the pIR1SAT expression vector (14), resulting in a pIR1SAT-*Ld*NTR construct.

Generation of LdBOB transgenic cell lines. Mid-log-phase *L. donovani* promastigotes (LdBOB) were transfected with either knockout or recovery constructs using the Human T-Cell Nucleofector kit and the Amaxa Nucleofector electroporator (program V-033). Following transfection, cells were allowed to grow for 16 to 24 h in modified M199 medium (11) with 10% fetal calf serum prior to appropriate drug selection (100 μg nourseothricin ml^{-1}, 50 μg hygromycin ml^{-1}, and 20 μg puromycin ml^{-1}). Cloned cell lines were generated by limiting dilution, maintained in selective medium, and removed from drug selection for one passage prior to experiments.

Southern blot analyses of transgenic *L. donovani* cell lines. The ORF and 5′ UTR of LdBOB *NTR* were amplified by PCR (using the primers listed in Table 1 for the cloning of *L. donovani* NTR [*Ld*NTR] and knockout constructs) with the PCR DIG Probe Synthesis kit (Roche). The resulting digoxigenin (DIG)-labeled products were used as probes. Samples of genomic DNA (5 μg) from wild-type (WT) and transgenic cell lines were digested with appropriate restriction endonucleases, and the digestion products were first separated on a 0.8% agarose gel and then transferred to a positively charged nylon membrane (Roche). The membrane was hybridized overnight in DIG Easy Hyb solution (Roche) at 42°C with the DIG-labeled *TRYS* ORF or 5′ UTR probes (2 μl of PCR product). Following hybridization, membranes were washed twice under low-stringency conditions (25°C, 5 min, SSC with 0.1% sodium dodecyl sulfate [SDS]) and twice under high-stringency conditions (68°C, 15 min, 0.5× SSC with 0.1% SDS), where 1× SSC comprises 150 mM sodium chloride and 50 mM sodium citrate (pH 7.0). Bound probe was detected by using the DIG immunological detection kit (Roche) according to the manufacturer's instructions.

Northern blot analyses of transgenic *L. donovani* cell lines. Total RNA was isolated from mid-log-phase *L. donovani* (LdBOB) promastigotes (1×10^8) by using the RNeasy Plus kit (Qiagen). Northern blot analysis of RNA samples was carried out by separating RNA (10 μg) on a 1% (wt/vol) agarose-formaldehyde gel and then transferring the RNA to a positively charged nylon membrane (Roche). The membrane was hybridized overnight in DIG Easy Hyb solution (Roche) at 68°C with a DIG-labeled *NTR* RNA probe, generated from the *NTR* ORF by using the DIG Northern starter kit (Roche). Following hybridization, membranes were washed, and bound probe was detected in a manner identical to that described above for Southern blots. Levels of NTR transcripts in each cell line were directly compared by densitometry using the ImageJ program.

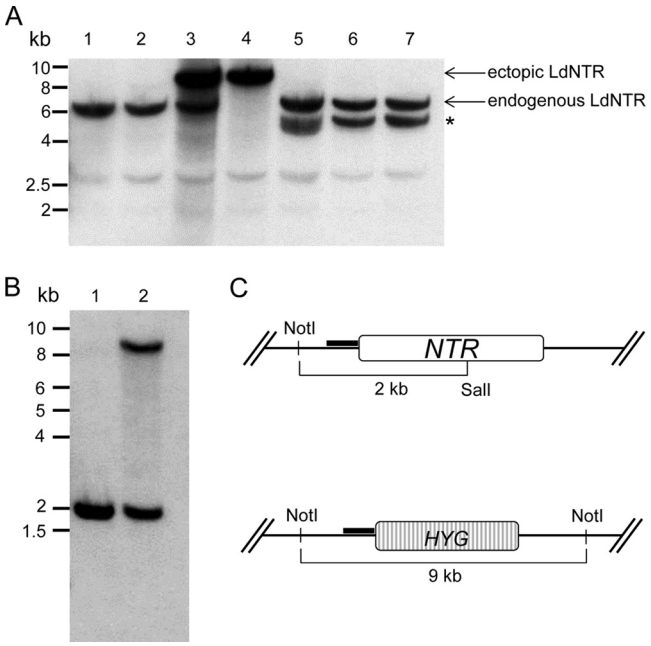


FIG 1 Genotypic analysis of WT, SKO, and rDKO cell lines. (A) Southern blot analysis of SalI-digested genomic DNA (~5 μg) from wild-type *L. donovani* (LdBOB) cells (lane 1), NTR single knockout (HYG) cells (lane 2), NTR single knockout (HYG) cells constitutively expressing NTR (lane 3), NTR double knockout (PAC and HYG) cells constitutively expressing NTR (lane 4), and "false" NTR double knockout (PAC and HYG) cells (lanes 5 to 7). The *NTR* ORF probe shows allelic *LdNTR* at 6.5 kb, the ectopic copy of *LdNTR* at ~9 kb, and a chromosomally rearranged copy of *NTR* at 5.5 kb (*). (B) Southern blot analysis of NotI-digested genomic DNA (~5 μg) from wild-type *L. donovani* (LdBOB) cells (lane 1) and NTR single knockout (HYG) cells (lane 2) probed with the 5′ UTR of *NTR*. (C) Structure of the *NTR* locus in NTR single knockout (HYG) cells. Black bars represent the 5′ UTR region upstream of the open reading frame of *NTR*, which was used as a probe in Southern blot analysis. NotI sites with expected fragment sizes are shown. The endogenous *NTR* gene contains a NotI site that results in a 2-kb band. Successful replacement of one allelic copy of the endogenous gene with *HYG*, which does not contain a NotI site, results in a 9-kb fragment.

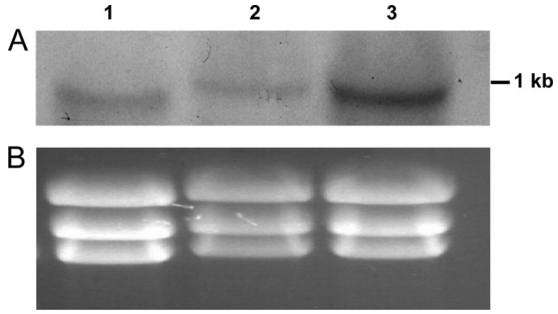


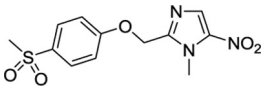
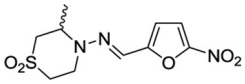
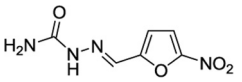
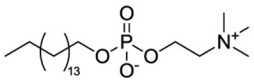
FIG 2 Analysis of NTR transcript levels by Northern blotting. (A) RNA samples (10 μg) were prepared from WT (lane 1), SKO (lane 2), and OE (lane 3) promastigotes and were analyzed by Northern blotting. Blots were hybridized with an RNA probe generated from the LdBOB *NTR* gene. (B) Ethidium bromide staining of RNA samples loaded on an agarose-formaldehyde gel prior to transfer.

RESULTS

Generation of NTR-complemented null mutants. The essentiality of *L. donovani* nitroreductase was assessed by classical gene replacement, where *NTR* is sequentially replaced with drug resistance genes. The first copy of the *NTR* gene was replaced with the hygromycin resistance gene (*HYG*, encoding hygromycin phosphotransferase) by homologous recombination and subsequent selection for hygromycin resistance, generating a single knockout (SKO) cell line (Fig. 1A and B). SKO promastigotes grew at the same rate in culture as homozygotes, and to the same cell density. Attempts were then made to create a null mutant by directly replacing the second allelic copy with the puromycin *N*-acetyltransferase gene (*PAC*). On four separate occasions, promastigotes resistant to both hygromycin and puromycin were recovered from transfections. However, Southern blot analysis of genomic DNA from multiple clones of these putative double knockout (DKO) cells revealed that they not only retained an endogenous copy of *NTR* but also had generated an additional gene copy, presumably by chromosomal rearrangement (Fig. 1A). Consequently, a rescued double knockout (rDKO) cell line was generated by introducing an ectopic copy of *NTR* into SKO promastigotes prior to replacing the second copy with *PAC*. This ectopic copy of *NTR* was inserted into the ribosomal DNA (rDNA) locus of the SKO cell line by using the pIR1SAT vector, which encodes a nourseothricin resistance gene (*SAT*), resulting in the constitutive expression of NTR. All clones of rDKO promastigotes were confirmed by Southern blotting to have lost both copies of their endogenous *NTR* genes (Fig. 1A). Our failure to directly replace both endogenous copies of the *NTR*, except in the presence of an ectopic copy of the gene, strongly suggests that the *NTR* gene is essential for the successful growth and survival of *L. donovani* promastigotes in culture.

Modulation of NTR levels in *L. donovani* alters sensitivity to nitro drugs. Modulation of nitroreductase levels within *Trypanosoma brucei* and *Trypanosoma cruzi* has been shown to directly affect sensitivity to nitro compounds *in vitro*, with reduced concentrations of the enzyme leading to nitro drug resistance (10). To determine whether this is also the case for *L. donovani*, the sensitivities of WT, SKO, and NTR-overexpressing (OE) promastigotes to a number of nitroheterocyclic compounds were assessed. First, modulation of NTR in the transgenic cell lines was confirmed by comparing *NTR* transcript levels by Northern blotting (Fig. 2). *NTR* transcript levels were 1.7-fold lower in SKO parasites and 4.2-fold higher in OE parasites than in WT parasites, as determined by densitometry. As expected, changes in the level of NTR in these cells correlated directly with their relative sensitivities to nifurtimox and nitrofurazone, nitrofuran compounds known to undergo NTR-catalyzed 2-electron reduction in trypanosomes (Table 2). Promastigotes overexpressing NTR were found to be 16- and 4-fold more sensitive than WT cells to nifurtimox and nitrofurazone, respectively. Conversely, SKO parasites were less sensitive to these compounds, with EC_{50}s of 7.2 and 2.5 μM for nifurtimox and nitrofurazone, respectively, compared to values of 5.0 and 1.4 μM for WT cells. Similar NTR level-dependent shifts in sensitivity were observed in cells treated with fexinidazole sulfone, the predominant active metabolite of the nitroimidazole fexinidazole *in vivo* (8, 15). While parasites overexpressing NTR were 18-fold more susceptible to fexinidazole sulfone than WT parasites, SKO promastigotes were marginally less sensitive (<2-fold [Table 2]). In contrast, there was no concomitant shift in the sensitivity of promastigotes to the alkyl phospholipid miltefosine. Thus, NTR plays a crucial role in the activation of fexini-

TABLE 2 Sensitivities of WT, SKO, and OE promastigotes to standard drugs and experimental nitro compounds

Compound	Structure	EC_{50}[a] (Hill slope) for *L. donovani*		
		WT	OE	SKO
Fexinidazole sulfone		7.2 ± 0.2 (5.4)	0.4 ± 0.01[b] (2.3)	12.6 ± 0.1[b] (2.3)
Nifurtimox		5.0 ± 0.05 (4.7)	0.32 ± 0.01[b] (2.8)	7.23 ± 0.08[b] (2.5)
Nitrofurazone		1.43 ± 0.02 (5.5)	0.35 ± 0.01[b] (2.4)	2.5 ± 0.02[b] (2.5)
Miltefosine		7.7 ± 0.1 (3.5)	8.16 ± 0.05 (3.8)	7.9 ± 0.2 (3.3)

[a] Expressed as micromolar concentrations. Results are weighted means and standard errors for at least two independent experiments.
[b] *P*, <0.0001 for comparison to the WT by Student's *t* test.

dazole and its metabolites in *L. donovani*, and NTR depletion within cells can lead to minor levels of resistance in promastigotes.

NTR modulation in intracellular amastigotes. In a manner identical to that seen with insect-stage promastigotes, the potency of fexinidazole sulfone for intracellular mammalian-stage amastigotes was materially altered by changes in cellular NTR levels. Metacyclic promastigotes (WT, SKO, and OE) were used to infect starch-elicited mouse peritoneal macrophages, and EC_{50}s were determined for each cell line following a 72-h incubation in varying concentrations of the drug (Fig. 3A). Intracellular amastigotes overexpressing NTR were approximately 9-fold more sensitive to fexinidazole sulfone (EC_{50}, 0.7 μM) than WT parasites (EC_{50}, 6.4 μM), while SKO parasites showed a <2-fold decrease in sensitivity to the drug (EC_{50}, 8.8 μM). The finding that loss of a single copy of the *NTR* gene leads to fexinidazole resistance in *L. donovani* is very much in keeping with the resistance mechanisms identified in laboratory-generated fexinidazole-resistant *T. brucei* cell lines (15). However, it should be noted that the EC_{99} of fexinidazole sulfone against intracellular SKO amastigotes (78.5 μM), calculated using Hill slopes, is not markedly different from that calculated for WT amastigotes (69.6 μM).

Loss of an allelic copy of *NTR* did not diminish the infectivity of SKO metacyclic promastigotes: the mean number of amastigotes per infected macrophage and the overall percentage of infected macrophages in untreated controls were comparable to those seen in WT-infected macrophage cultures (Fig. 3B and C). However, parasites overexpressing the NTR did appear to lose "fitness." Both the number of OE amastigotes per infected macrophage and the percentage of macrophages infected with the OE cell line were significantly lower than those for the WT (*P*, 0.04 and 0.03, respectively).

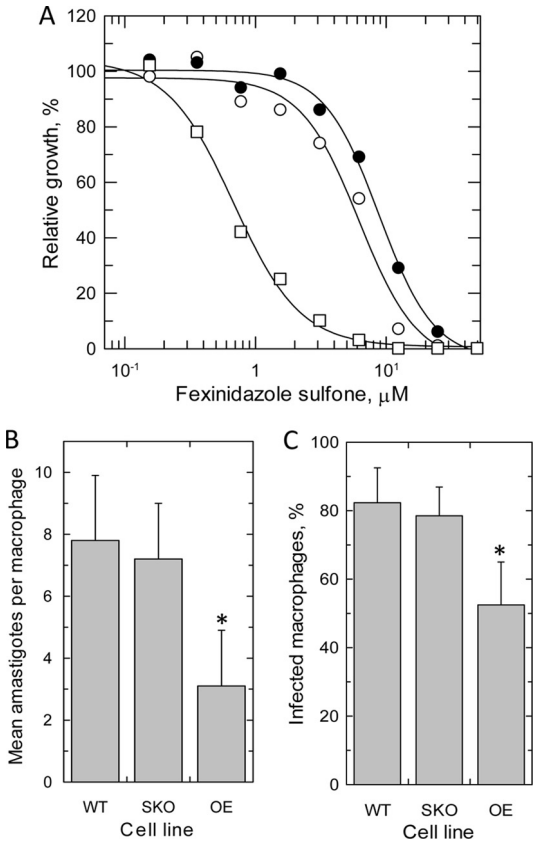


FIG 3 Effects of NTR modulation on susceptibility to fexinidazole sulfone. The susceptibilities of amastigotes cultured in starch-elicited mouse peritoneal macrophages were determined. (A) The EC_{50}s of fexinidazole sulfone against WT (open circles), SKO (HYG) (filled circles), and OE (open squares) parasites were determined. EC_{50}s of 6.4 ± 0.9, 8.8 ± 0.7, and 0.7 ± 0.06 μM were found for fexinidazole sulfone against the WT, SKO, and OE cell lines, respectively. (B) The mean number of WT, SKO, or OE amastigotes infecting mouse peritoneal macrophages in untreated control cultures was determined. Differences in the number of WT versus OE amastigotes infecting macrophages were confirmed as statistically significant ($P = 0.04$) by using an unpaired Student *t* test (*). (C) The percentage of macrophages infected with WT, SKO, or OE amastigotes in untreated control cultures was determined. Differences in the percentage of macrophages infected with WT versus OE amastigotes were confirmed as statistically significant ($P = 0.03$) by using an unpaired Student *t* test (*). All the data are means for triplicate measurements.

DISCUSSION

Our inability to knock out both allelic copies of *NTR*, except in the presence of an ectopic copy of the gene, provides strong evidence that the endogenous function of NTR is essential for the viability of *L. donovani* promastigotes. This is also true for the African trypanosome: *NTR* could not be deleted from bloodstream-form *T. brucei* parasites (10). In the case of the South American trypanosome *T. cruzi*, *NTR*-null mutants of the epimastigote (insect

stage) are unable to differentiate into infectious metacyclic trypomastigotes and do not infect mammalian cells (10). Thus, NTR activity appears to be an absolute requirement for the virulence of trypanosomatids in their mammalian hosts. The biological functions of trypanosomal NTRs remain to be determined. However, there is some evidence that these enzymes may play a role in ubiquinone metabolism. Trypanosomal NTRs share several features with mitochondrial NADH dehydrogenases (10), enzymes known to catalyze the transfer of electrons from NADH to ubiquinone, leading to the generation of ubiquinol (16). In addition, a recent genomewide RNA interference (RNAi) target-sequencing screen of *T. brucei* demonstrated a link between nitro drug action, ubiquinone biosynthesis, and nitroreductases (17). While further studies will be required in order to pinpoint the precise essential biological function of the trypanosomal NTRs, it is clear that these enzymes play a crucial role in the activation of several nitro drugs. The essentiality of these enzymes in all three "Tritryp" parasites also makes them attractive targets for the development of novel chemotherapeutic agents that do not undergo reductive activation.

Loss of a single chromosomal copy of the *L. donovani NTR* gene resulted in parasites that were mildly resistant to several nitroheterocyclic compounds, including the predominant *in vivo* metabolite of the antileishmanial drug candidate fexinidazole. These data, combined with the pronounced hypersensitivity demonstrated by promastigotes overexpressing NTR, confirm that the *Leishmania* NTR plays a pivotal role in the activation of nitro drugs in general, and in the mechanism of action of fexinidazole in particular. Recent studies have shown that trypanosomal nitroreductases catalyze the sequential 2-electron reduction of nifurtimox, resulting in the generation of a cytotoxic, unsaturated open-chain nitrile derivative (9). It remains to be seen whether fexinidazole and its metabolites undergo a similar 2-electron reduction catalyzed by the *Leishmania* nitroreductase. Importantly, these findings also illustrate a mechanism by which clinical resistance to fexinidazole could emerge. Reliance on a single enzyme for prodrug activation may leave drugs such as fexinidazole vulnerable to the emergence of drug resistance. However, our failure to generate NTR-null promastigotes clearly suggests that there is selective pressure for *Leishmania* parasites to retain some NTR activity. This pressure may have the added advantage of limiting the maximum levels of fexinidazole resistance achievable, with SKO promastigotes less than 2-fold more resistant to the drug. In recent studies with *T. cruzi*, laboratory-generated benznidazole-resistant parasites were found to have lost a single copy of the *NTR* gene, and mutations arising in the remaining copy of the gene abolished enzyme activity (18). However, these "functional null" parasites demonstrated such reduced levels of virulence that the capacity for such highly drug resistant *T. cruzi* parasites to spread within the population would be severely compromised. At this stage in our studies, we cannot exclude the possibility that similar mutations within the *NTR* gene of *L. donovani* may lead to high levels of clinically relevant fexinidazole resistance. In order to investigate this possibility, as well as the potential for non-NTR-related mechanisms of resistance, we are currently generating fexinidazole-resistant *L. donovani* cell lines.

With a limited number of drugs available for the treatment of visceral leishmaniasis, effective policies to prevent the development of resistance to new and existing antileishmanial drugs are essential. One such strategy involves the use of drugs in combination, with the rationale that the likelihood of developing resistance to a single agent is relatively high, but the likelihood of developing resistance to two compounds is significantly lower (19). The use of drug combinations to circumvent resistance has been successful with antimalarials (19), and this approach is now being seriously considered for the treatment of visceral leishmaniasis. Indeed, a recent trial directly compared the efficacies of three potential short-course combination treatments with that of the standard monotherapy in India (20). The results of that study confirm that these combination treatments for visceral leishmaniasis are efficacious and safe and may well reduce the emergence of drug-resistant parasites. With the progression of fexinidazole into clinical development for use against visceral leishmaniasis, work to identify an appropriate partner drug for this nitroimidazole is under way.

ACKNOWLEDGMENTS

This work was supported by grants (079838, 077705, and 083481) to A.H.F. from the Wellcome Trust (http://www.wellcome.ac.uk).

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