

# An Essential Type I Nitroreductase from *Leishmania major* Can Be Used to Activate Leishmanicidal Prodrugs

Received for publication, June 18, 2013, and in revised form, July 29, 2013. Published, JBC Papers in Press, August 14, 2013, DOI 10.1074/jbc.M113.494781

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**Background:** Leishmaniasis is a tropical disease prevalent in developing countries.

**Results:** *Leishmania major* type I nitroreductase (LmNTR) metabolizes nitroaromatic/quinone substrates, an activity essential for parasite viability.

**Conclusion:** LmNTR exhibits biochemical characteristics of its bacterial counterparts, and its activity is essential for parasite growth.

**Significance:** LmNTR can be exploited as a prodrug activation enzyme or as a target for inhibitor design in drug development.

Nitroaromatic prodrugs are used to treat a range of microbial infections with selectivity achieved by specific activation reactions. For trypanosomatid parasites, this is mediated by type I nitroreductases. Here, we demonstrate that the causative agent of leishmaniasis, *Leishmania major*, expresses an FMN-containing nitroreductase (LmNTR) that metabolizes a wide range of substrates, and based on electron donor and acceptor preferences, it may function as an NADH:quinone oxidoreductase. Using gene deletion approaches, we demonstrate that this activity is essential to *L. major* promastigotes, the parasite forms found in the insect vector. Intriguingly, *LmNTR*<sup>+/-</sup> heterozygote promastigote parasites could readily differentiate into infectious metacyclic cells but these were unable to establish infections in cultured mammalian cells and caused delayed pathology in mice. Furthermore, we exploit the LmNTR activity evaluating a library of nitrobenzylphosphoramidate mustards using biochemical and phenotypic screens. We identify a subset of compounds that display significant growth inhibitory properties against the intracellular parasite form found in the mammalian hosts. The leishmanicidal activity was shown to be LmNTR-specific as the *LmNTR*<sup>+/-</sup> heterozygote promastigotes displayed resistance to the most potent mustards. We conclude that LmNTR can be targeted for drug development by exploiting its prodrug activating property or by designing specific inhibitors to block its endogenous function.

Insect-transmitted protozoan parasites belonging to the genus *Leishmania* are responsible for a spectrum of diseases

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known as leishmaniasis. These infections are endemic in 88 countries around the world with ~350 million people living in “at risk” areas (1). Recently, as a result of military activity, population migration, modern medical practices, intravenous drug usage, and global warming, leishmaniasis has emerged as a problem in nonendemic areas (2–4).

A vaccine for visceral leishmaniasis is in phase I trials, but currently drugs are the only option available to treat leishmanial infections. Use of the frontline antimonial-based therapies is problematic as they are toxic, clinical resistance is on the rise, and they often require medical supervision to administer (5). Recent progress has been made in developing new leishmanicidal agents with several compounds such as amphotericin B, paromomycin, and miltefosine coming to market (1). However, there are issues associated with the use of these as they are expensive and require medical administration with some having teratogenic and other unwanted toxicity problems (6). Against this backdrop, the development of new cost-effective treatments is a priority, but given that leishmaniasis mainly affects people living in developing countries, these infections are not deemed commercially attractive by pharmaceutical companies. As a result, leishmaniasis is largely neglected in terms of drug development (7).

Nitroaromatic compounds encompass a wide range of compounds characterized by at least one nitro group attached to an aromatic ring (8). They have been used in medicine predominantly as antimicrobial agents, but concerns over their mutagenicity have led to many being withdrawn in Europe and the United States (9–11). However, it is now apparent that several nitro-based compounds are not as toxic as initially thought (12–14) with retrospective analysis of nitrofurantoin clinical trial data coupled with cost evaluation resulting in calls for the reinstatement of this prodrug as a treatment for uncomplicated urinary tract infections (15). This resurgence of interest has led to several nitroaromatic compounds undergoing evaluation for treatments of infectious organisms, including PA-824 and OPC-67683 against *Mycobacterium tuberculosis*, nitazoxanide against

*Giardia*, *Cryptosporidium*, and hepatitis C (16–18), and fexinidazole against *Trypanosoma brucei* and *Leishmania donovani* (19, 20), whereas others such as SN23862, CB1954, and nifurtimox have emerged as possible anti-cancer therapies (21–23).

Most antimicrobial nitroaromatic compounds function as prodrugs and must undergo activation before producing their cytotoxic activity, a process mediated by nitroreductases (NTRs).<sup>5</sup> Based on oxygen sensitivity and flavin cofactors, NTRs can be broadly divided into two groups (24). Type I NTRs utilize NAD(P)H as an electron donor, transferring reducing equivalents via an FMN cofactor to the substrate in a series of sequential two-electron reduction events. This nitroreduction does not involve oxygen and does not result in the production of reactive oxygen species, an activity said to be “oxygen-insensitive.” In contrast, the type II NTRs contain FAD or FMN as cofactor and catalyze the one-electron reduction of the substrate-conserved nitro group to generate a nitro radical. This radical reacts with oxygen to produce superoxide anions with the subsequent regeneration of the original nitro compound; type II NTRs is said to be “oxygen-sensitive” (25). The difference in NTR distribution is believed to underlie the specificity of most antimicrobial nitroaromatic prodrugs with type I NTRs found mainly in bacteria and absent from most eukaryotes, with a subset of fungi and protozoan parasites being the exceptions (26–28). Although some mammalian enzymes, such as NAD(P)H quinone oxidoreductase 1 and nitric-oxide synthase, can mediate two-electron reduction reactions under aerobic conditions, type II NTR activities predominate in most cell types (29).

The aim of this study was to characterize the type I NTR expressed by *Leishmania major*, the pathogen responsible for cutaneous leishmaniasis, and to analyze the role this enzyme plays in the parasite. Utilizing the activity, we then conducted biochemical and phenotypic screens using a library of nitrobenzylphosphoramidate mustard (NBPM) compounds to identify potential leishmanicidal agents targeting the intracellular mammalian form of the pathogen.

## EXPERIMENTAL PROCEDURES

**Compounds**—Nitrofurazone, nitrofurantoin, CB1954, coenzyme Q1, and duroquinone were purchased from Sigma. Benznidazole and nifurtimox were provided by Simon Croft (London School of Hygiene and Tropical Medicine), and metronidazole was a gift from Nubia Boechat (Far Manguinhos, Rio de Janeiro). The NBPM structures (Tables 1 and 2) were fully characterized using NMR and MS, and their purity was judged to be >90%; most were >95% based on LC-MS analysis (30–33).

**Parasite Culture and Genetic Manipulation**—*L. major* (MHOM/IL/80/Friedlin) promastigote form parasites were grown at 27 °C in M199 medium (Invitrogen) supplemented with 4 mM sodium bicarbonate, 40 mM HEPES, pH 7.4, 0.1 mM adenine, 0.005% (w/v) hemin, 2.5 units ml<sup>-1</sup> penicillin, 25 μg ml<sup>-1</sup> streptomycin, and 20% (v/v) fetal calf serum. Transformed parasites were grown in this medium containing G418

**TABLE 1**  
Structure of cyclic nitrobenzylphosphoramidate mustards

compound	structure	diastereomer
Compounds based on A		
LH3	X=O; Y=NH	cis
LH4	X=O; Y=NH	trans
LH5	X=NH; Y=O	cis
LH6	X=NH; Y=O	trans
LH12	X=NH; Y=NH	cis
LH13	X=NH; Y=NH	trans
Compounds based on B		
LH8	X=O; Y=NH	cis
LH9	X=O; Y=NH	trans

**TABLE 2**  
Structure of acyclic nitrobenzylphosphoramidate mustards

compound	structure
LH7	R <sub>3</sub> =NO <sub>2</sub> ; X=P; Y=NH <sub>2</sub> ; R <sub>1</sub> =R <sub>2</sub> =R <sub>4</sub> =R <sub>5</sub> =Z=H
LH14	R <sub>3</sub> =NO <sub>2</sub> ; X=P; Y=NH <sub>2</sub> ; Z=CH <sub>3</sub> ; R <sub>1</sub> =R <sub>2</sub> =R <sub>4</sub> =R <sub>5</sub> =H
LH15	R <sub>3</sub> =NO <sub>2</sub> ; X=P; Y=NH <sub>2</sub> ; R <sub>1</sub> =R <sub>2</sub> =R <sub>3</sub> =R <sub>4</sub> =Z=H
LH16	R <sub>4</sub> =NO <sub>2</sub> ; X=P; Y=NH <sub>2</sub> ; R <sub>1</sub> =R <sub>2</sub> =R <sub>3</sub> =R <sub>5</sub> =Z=H
LH17	R <sub>3</sub> =NO <sub>2</sub> ; R <sub>5</sub> =OCH <sub>3</sub> ; X=P; Y=NH <sub>2</sub> ; R <sub>1</sub> =R <sub>2</sub> =R <sub>4</sub> =Z=H
LH18	R <sub>3</sub> =NO <sub>2</sub> ; R <sub>4</sub> =OCH <sub>3</sub> ; X=P; Y=NH <sub>2</sub> ; R <sub>1</sub> =R <sub>2</sub> =R <sub>3</sub> =Z=H
LH19	R <sub>3</sub> =NO <sub>2</sub> ; R <sub>4</sub> =CH <sub>3</sub> ; X=P; Y=NH <sub>2</sub> ; R <sub>1</sub> =R <sub>2</sub> =R <sub>5</sub> =Z=H
LH24	R <sub>3</sub> =NO <sub>2</sub> ; X=P; Y=NH <sub>2</sub> ; Z=CH <sub>3</sub> ; R <sub>1</sub> =R <sub>2</sub> =R <sub>4</sub> =R <sub>5</sub> =H
LH27	R <sub>3</sub> =NO <sub>2</sub> ; X=C; R <sub>1</sub> =R <sub>2</sub> =R <sub>4</sub> =R <sub>5</sub> =Z=H
LH31	R <sub>2</sub> =F; R <sub>3</sub> =NO <sub>2</sub> ; X=P; Y=NH <sub>2</sub> ; R <sub>1</sub> =R <sub>4</sub> =R <sub>5</sub> =Z=H
LH32	R <sub>1</sub> =F; R <sub>3</sub> =NO <sub>2</sub> ; X=P; Y=NH <sub>2</sub> ; R <sub>2</sub> =R <sub>4</sub> =R <sub>5</sub> =Z=H
LH33	R <sub>1</sub> =CF <sub>3</sub> ; R <sub>3</sub> =NO <sub>2</sub> ; X=P; Y=NH <sub>2</sub> ; R <sub>2</sub> =R <sub>4</sub> =R <sub>5</sub> =Z=H
LH34	R <sub>1</sub> =Cl; R <sub>3</sub> =NO <sub>2</sub> ; X=P; Y=NH <sub>2</sub> ; R <sub>2</sub> =R <sub>4</sub> =R <sub>5</sub> =Z=H
LH37	R <sub>1</sub> =R <sub>5</sub> =F; R <sub>3</sub> =NO <sub>2</sub> ; X=P; Y=NH <sub>2</sub> ; R <sub>2</sub> =R <sub>4</sub> =Z=H

(20 μg ml<sup>-1</sup> on agar plates, 40 μg ml<sup>-1</sup> in broth), blasticidin (10 μg ml<sup>-1</sup>), or puromycin (20 μg ml<sup>-1</sup>). *L. major* metacyclic form parasites were harvested from promastigote cultures in the stationary phase of growth (7–10-day-old cultures) following agglutination of promastigote parasites with peanut lectin (Sigma) (34). The metacyclics were used to infect differentiated human acute monocytic leukemia (THP-1) cells at a ratio of 20 parasites per mammalian cell. The *L. major* infected monolayers were incubated overnight at 37 °C under a 5% (v/v) CO<sub>2</sub> atmosphere in mammalian growth medium and then washed with RPMI 1640 to remove residual parasites. *L. major* amastigote parasites were maintained in differentiated THP-1 cells at 37 °C under a 5% (v/v) CO<sub>2</sub> atmosphere in mammalian growth medium.

For DNA transfection, *L. major* promastigotes (2 × 10<sup>7</sup>) in the logarithmic phase of growth were pelleted, resuspended in 100 μl of human T-cell Nucleofector<sup>®</sup> solution (Lonza AG) containing 5 μg of purified DNA, and electroporated using program U-033 on the Nucleofector<sup>™</sup> (Lonza AG). Cells were then transferred to M199 growth medium and allowed to recover for 20–24 h at 27 °C. Parasites were pelleted, resus-

<sup>5</sup> The abbreviations used are: NTR, type I nitroreductase; BLA, blasticidin resistance gene cassette; LmNTR, *L. major* type I nitroreductase; NBPM, nitrobenzylphosphoramidate mustard; PAC, puromycin resistance gene cassette; TcNTR, *T. cruzi* type I nitroreductase.

## Characterization of a *Leishmania* Nitroreductase

pended in 100  $\mu\text{l}$  of promastigote growth medium, and then spread onto promastigote growth medium solidified with 1% (w/v) agar containing 1.2  $\mu\text{g ml}^{-1}$  bioppterin (Sigma) and the appropriate selective agent. The plates were incubated at 27 °C until colonies appeared. Colonies were then transferred into promastigote growth medium containing the appropriate selective agent and grown as described above.

**Mammalian Cell Culturing**—The human acute monocytic leukemia cell line (THP-1) was grown at 37 °C under a 5% (v/v) CO<sub>2</sub> atmosphere in RPMI 1640 medium (PAA Laboratories Ltd.) supplemented with 2 mM pyruvate, 2 mM sodium glutamate, 2.5 units ml<sup>-1</sup> penicillin, and 2.5  $\mu\text{g ml}^{-1}$  streptomycin, 20 mM HEPES, pH 7.4, and 10% (v/v) fetal calf serum. Differentiation of THP-1 toward macrophage-like cells was carried out using phorbol 12-myristate 13-acetate (20 ng ml<sup>-1</sup>) (Sigma) (35).

**In Vivo Studies**—All animal experiments were conducted under license in accordance with regulations from United Kingdom Home Office. Female BALB/c mice were infected by subcutaneous injection of  $2 \times 10^7$  purified metacyclic parasites in 100  $\mu\text{l}$  of RPMI 1640 medium without serum into their shaved rump. Mice were monitored for lesion pathology, and when observed, the diameter of each lesion was measured in two dimensions at a rectangular angle with digital calipers (Jencons Scientific Ltd.), and the mean diameter was calculated. Three mice were examined for each treatment.

**Antiproliferative Assays**—All assays were performed in a 96-well plate format. *L. major* promastigote parasites ( $5 \times 10^5$  ml<sup>-1</sup>) or differentiated THP-1 cells ( $2.5 \times 10^4$  ml<sup>-1</sup>) were seeded in 200  $\mu\text{l}$  of growth medium containing different concentrations of nitroaromatic agent. After incubation at 27 °C for 5 days (*L. major*) or at 37 °C for 3 days (THP-1), 2.5  $\mu\text{g}$  of resazurin (20  $\mu\text{l}$  of 0.125  $\mu\text{g ml}^{-1}$  stock in phosphate-buffered saline) was added to each well, and the plates were incubated for a further 8–16 h. Cell densities were determined by monitoring the fluorescence of each culture using a Gemini Fluorescent Plate Reader (Molecular Devices Ltd., Wokingham, UK) at an excitation wavelength of 530 nm, emission wavelength of 585 nm, and a filter cutoff at 550 nm, and the drug concentration that inhibited cell growth by 50% (IC<sub>50</sub>) was established.

Growth inhibition of luciferase expressing *L. major* amastigotes was monitored as follows. THP-1 cells seeded at  $2.5 \times 10^4$  ml<sup>-1</sup> in 200  $\mu\text{l}$  in growth medium containing phorbol 12-myristate 13-acetate (20 ng ml<sup>-1</sup>) were incubated at 37 °C in a 5% (v/v) CO<sub>2</sub> atmosphere for 3 days. Macrophage monolayers were copiously washed with mammalian growth medium and then infected with purified *L. major* metacyclic cells ( $5 \times 10^5$  cells ml<sup>-1</sup>) resuspended in 200  $\mu\text{l}$  of mammalian growth medium. Following incubation overnight at 37 °C in a 5% (v/v) CO<sub>2</sub> atmosphere, the cultures were washed twice in growth medium to remove noninternalized parasites, and the supernatant was replaced with fresh growth medium containing drug. Drug-treated infections were incubated for a further 3 days at 37 °C under 5% (v/v) CO<sub>2</sub>. The growth medium was then removed, and the cells were lysed in 50  $\mu\text{l}$  of cell culture lysis reagent (Promega). Activity was then measured using the luciferase assay system (Promega), and light emission was measured on a  $\beta$ -plate counter (PerkinElmer Life Sciences). The luminescence

was proportional to the number of live cells. The IC<sub>50</sub> value for each compound was then established.

**Plasmids**—The integrative *L. major* luciferase vector pLmRIX-luc was generated by sequentially cloning the *L. major* 5'-spacer/promoter rRNA and 3'-spacer rRNA regions on either side of an expression cassette containing the luciferase reporter and neomycin resistance genes. The *TcMPX* polypyrimidine tract/splice leader addition site sequence was inserted between the *L. major* 5'-spacer/promoter rRNA and the luciferase gene to provide the genetic elements required for processing the reporter mRNA transcript. The resultant construct, pLmRIX-Luc, was linearized prior to electroporation into *L. major* promastigotes.

The vectors used to delete *LmNTR*, pKO-*LmNTR*-PAC, and pKO-*LmNTR*-BLA were generated by sequentially cloning *LmNTR* flanking sequences on either side of a puromycin or blasticidin resistance cassette (see Fig. 3). The constructs were linearized and then electroporated into *L. major* promastigotes.

A DNA fragment encoding for the catalytic domain of *L. major* NTR (LmNTR) was amplified from genomic DNA with the primers *ggatcc*CTCGACGCCGTCGAGGCCGTCG and *aagctt*CTAGAACTTGTTCACCGCAC, with lowercase italics indicating restriction sites incorporated into the primers to facilitate cloning. The fragment was digested with BamHI/HindIII and then cloned into the corresponding sites of the vector pTrcHisC (Invitrogen) to form the plasmid pTrcHisC-LmNTR. Site-directed mutagenesis was carried out using a Stratagene QuikChange mutagenesis kit (Agilent Technologies), with pTrcHisC-LmNTR as template. Amplifications were performed in accordance with the manufacturer's instructions using primers (Eurofins) to generate the desired mutation. The forward primer sequence was R96A (GCCGTCGTGCGC-GACGCGTGACGTCGCCGGCAG). The relevant substitution site, incorporating the required base change, is underlined.

**Protein Purification**—An overnight culture of *Escherichia coli* BL21(+) containing the expression plasmid was diluted 1:50 in NZCYM (Sigma) medium containing 100  $\mu\text{g ml}^{-1}$  ampicillin and grown for 2–4 h at 37 °C. The culture was transferred to 16 °C for 30 min, and the protein expression was induced by addition of isopropyl  $\beta$ -D-thiogalactopyranoside to a final concentration of 1 mM. The culture was then incubated overnight at 16 °C. The cells were harvested then lysed in Buffer A (50 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 7.8, 500 mM NaCl) containing 1 mg ml<sup>-1</sup> lysozyme, 10  $\mu\text{g ml}^{-1}$  DNase, 10  $\mu\text{g ml}^{-1}$  RNase, 1% (w/v) CHAPS, and a mixture of protease inhibitors (Roche Applied Science). The clarified supernatant was then applied to a pre-packed nickel-nitrilotriacetic acid (Qiagen) column, and the column was copiously washed with Buffer A and with Buffer A containing 50 and 100 mM imidazole. Recombinant protein was eluted off the column using Buffer A supplemented with 500 mM imidazole and 1% (w/v) CHAPS. Glycerol was then added to a final volume of 20% (v/v), and aliquots were stored at -80 °C. Protein concentration was determined by the BCA assay system (Thermo Fisher Scientific, Cramlington, UK), and the purity level was confirmed by SDS-PAGE.

**Enzyme Activity**—LmNTR activity was measured spectrophotometrically using several electron acceptors as substrate. A standard reaction (1 ml) containing 50 mM Tris-Cl, pH 7.5, 100  $\mu\text{M}$  NADH, and 100  $\mu\text{M}$  electron acceptor was incubated at

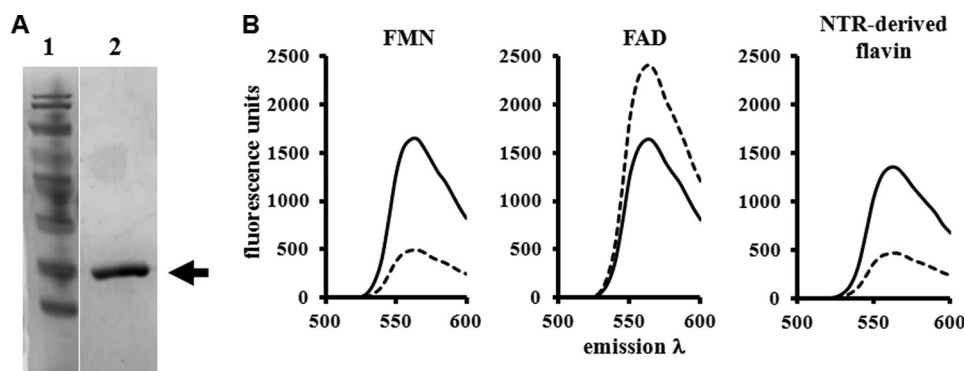


FIGURE 1. **Leishmanial NTRs contain FMN as a cofactor.** *A*, Coomassie-stained SDS-polyacrylamide gel (10%) containing purified, recombinant LmNTR (lane 2). Lane 1, size standards. *B*, fluorescence spectra of FMN and FAD (both 50  $\mu\text{M}$ ) and of supernatant from boiled and purified recombinant LmNTR (0.5 mg) at pH 7.6 (solid line) and pH 2.2 (dashed line) with excitation at 450 nm and emission between 480 and 600 nm. All the fluorescence analyses were carried out in triplicate; the profiles are derived from the mean values.

room temperature for 5 min. The background reaction rate was determined, and the assay was initiated by addition of the LmNTR (35  $\mu\text{g}$ ). For reactions containing nitroimidazoles, nitrobenzylphosphoramides, or most quinones, activity was measured by following the change in absorbance at 340 nm corresponding to NADH oxidation ( $\epsilon = 6,200 \text{ M}^{-1} \text{ cm}^{-1}$ ), although for assays involving nitrofurans, the direct reduction of the substrate itself was monitored at 435 nm for nifurtimox ( $\epsilon = 18,000 \text{ M}^{-1} \text{ cm}^{-1}$ ) or at 400 nm for nitrofurazone and nitrofurantoin ( $\epsilon = 12,000$  and  $15,000 \text{ M}^{-1} \text{ cm}^{-1}$ , respectively (36)). For CB1954, activity was monitored by following the change in absorbance at 420 nm, corresponding to production of the hydroxylamine ( $\epsilon = 1200 \text{ M}^{-1} \text{ cm}^{-1}$  (37)). Data were evaluated by nonlinear regression analysis using GraphPad Prism 5 (GraphPad Software).

**Flavin Characterization**—The flavin cofactor bound to LmNTR was established by determining the fluorescence spectrum in acidic and neutral buffers (36, 38). Purified protein was desalted and boiled for 5 min. In a total volume of 100  $\mu\text{l}$ , clarified supernatants (90  $\mu\text{l}$ ) containing 0.5 mg of NTR were mixed with 10  $\mu\text{l}$  of 50 mM  $\text{NaH}_2\text{PO}_4$ , pH 7.6, or 1 M HCl (final pH 2.2). The fluorescence profile for each treatment was then determined using a Gemini Fluorescent Plate Reader (Molecular Devices Ltd) with an excitation  $\lambda = 450 \text{ nm}$  and an emission  $\lambda = 480\text{--}600 \text{ nm}$ . The resultant patterns were compared with profiles obtained using FMN and FAD standards.

## RESULTS

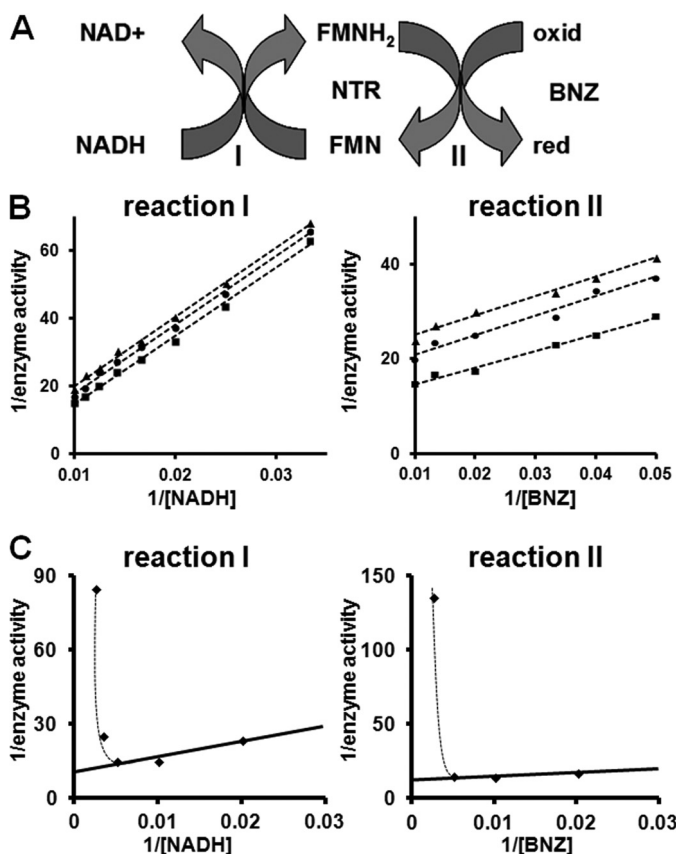
***L. major* Expresses a Functional Type I Nitroreductase**—Analysis of the *L. major* genome database identified a 972-bp open reading frame located on chromosome 5 with the potential to encode for a 34.7-kDa enzyme related to the type I nitroreductase family of proteins (LmNTR; GenBank<sup>TM</sup> accession number XP\_001687543). Full-length LmNTR is 90% identical to homologues present in *Leishmania mexicana* (CBZ23423) and *Leishmania infantum* (CAM65376) NTRs and 75% to *Leishmania braziliensis* NTR (CAM36899) and has 50% identity to the trypanosomal enzymes TcNTR (XP\_810645) and *T. brucei* type I nitroreductase (TbNTR) (XP\_846343). When compared with bacterial counterparts such as *E. coli* nfsB (NP\_415110) and *Bacillus cereus* NAD(P)H nitroreductase (NP\_832770), the highest level of sequence identity was

around 20%. Based on sequence, LmNTR can be divided into two regions. The amino-terminal portion (residues 1–86), absent from prokaryotic sequences, is predicted by SignalP, PSORT II, and iPSORT algorithms to contain a mitochondrial targeting signal (data not shown), although the remainder of the enzyme (residues 87–323) constitutes the catalytic domain.

To evaluate whether LmNTR could function as a nitroreductase, the DNA sequence encoding for the putative catalytic domain (residues 87–323) was expressed as a His-tagged protein in *E. coli*; attempts to express full-length recombinant LmNTR failed to generate soluble protein. The recombinant enzyme could be readily purified after one round of affinity chromatography with elutions containing the purified His-tagged LmNTR having a yellow coloration, indicative of a flavoprotein (Fig. 1A). To identify the nature of the flavin and investigate NTR/cofactor interaction, recombinant LmNTR was boiled, and the fluorescence profile of clarified supernatants under neutral and acidic pH values was analyzed (Fig. 1B) (36, 38). Denaturation resulted in release of the flavin from the enzyme, indicating that the cofactor had a noncovalent association with the protein backbone (39–41), whereas the fluorescence profiles under neutral and acidic pH values identified FMN as the flavin moiety (Fig. 1B); at neutral pH, and excitation at 450 nm, flavin derived from the parasite enzyme had a peak emission around 560 nm, a signal quenched in acidic buffers, typical of FMN and distinct from FAD. In the trypanosomal enzyme TcNTR, an arginine located in the RX(S/T/A)X(R/K) motif has been shown to play a key role in cofactor binding with this region and the residue conserved in LmNTR (42). To evaluate the importance of this arginine to LmNTR activity, it was converted to alanine (R96A). Expression of the His-tagged mutant protein in *E. coli* resulted in recombinant protein but at levels considerably lower than that obtained with the wild type enzyme. The mutated enzyme remained in insoluble fractions despite several attempts to optimize expression and purification conditions (data not shown).

To investigate its substrate specificity, His-tagged LmNTR activity was monitored under aerobic conditions by following NADH or NADPH oxidation at 340 nm or, when a compound's absorbance spectra precluded this, by following reduction of the substrate itself (Fig. 2A). This demonstrated that the recom-

## Characterization of a Leishmanial Nitroreductase



**FIGURE 2. Investigation of the kinetic properties of leishmanial type I nitroreductase toward benznidazole.** *A*, postulated scheme for the *L. major* type I nitroreductase-mediated reduction of benznidazole (*BNZ*) by using NADH as an electron donor. *Red* represents the reduced form, and *oxid* represents the oxidized form of benznidazole. The oxidized (*FMN*) and reduced (*FMNH<sub>2</sub>*) forms of the flavin cofactor are indicated. *B*, left panel, interaction of LmNTR with NADH (*reaction I*). Activity was assayed by monitoring the oxidation of NADH (30–100  $\mu\text{M}$ ) in the presence of benznidazole (50  $\mu\text{M}$  ( $\blacktriangle$ ), 75  $\mu\text{M}$  ( $\bullet$ ), and 100  $\mu\text{M}$  ( $\blacksquare$ )) and His-tagged LmNTR (35  $\mu\text{g}$ ). Right panel, interaction of LmNTR with benznidazole (*reaction II*). Activity was assayed by monitoring the oxidation of NADH (70  $\mu\text{M}$  ( $\blacktriangle$ ), 80  $\mu\text{M}$  ( $\bullet$ ), and 100  $\mu\text{M}$  ( $\blacksquare$ )) in the presence of benznidazole (20–100  $\mu\text{M}$ ) and His-tagged LmNTR (35  $\mu\text{g}$ ). *C*, inhibition of LmNTR activity by high concentrations of NADH or benznidazole. In reaction I, a fixed concentration of benznidazole (100  $\mu\text{M}$ ) was reduced by His-tagged LmNTR (35  $\mu\text{g}$ ) using various concentrations of NADH (50–400  $\mu\text{M}$ ). At high NADH levels (>200  $\mu\text{M}$ ), substrate inhibition was observed. In reaction II, a fixed concentration of NADH (100  $\mu\text{M}$ ) was oxidized by His-tagged LmNTR (35  $\mu\text{g}$ ) using various concentrations of benznidazole (50–400  $\mu\text{M}$ ). At benznidazole concentrations above 200  $\mu\text{M}$ , substrate inhibition was observed. All assays were initiated by the addition of the parasite enzyme. LmNTR activity is expressed as micromoles of NADH oxidized per  $\text{min}^{-1}$  mg of protein $^{-1}$ , whereas [NADH] and [benznidazole] are expressed in micromolar.

binant leishmanial enzyme functioned as typical type I NTR, able to catalyze the reduction of a wide range of nitroaromatics and quinones in an oxygen-insensitive fashion (Table 3). When the specificity constants ( $k_{\text{cat}}/K_m$ ) generated by these substrates were compared, LmNTR showed a preference for quinone compounds; the  $k_{\text{cat}}/K_m$  values exhibited by His-tagged LmNTR toward the ubiquinone 5 ( $2.0 \times 10^4$ ) was an order of magnitude greater than for CB1954 ( $4.6 \times 10^3$ ) and benznidazole ( $2.5 \times 10^3$ ). Interestingly, recombinant LmNTR could use both NADPH and NADH as electron donors, although when using fixed concentrations of benznidazole (100  $\mu\text{M}$ ) and NADPH or NADH (100  $\mu\text{M}$ ), activity values indicated a preference for NADH. Under these conditions, LmNTR had a specific

**TABLE 3**  
Substrate specificity of LmNTR

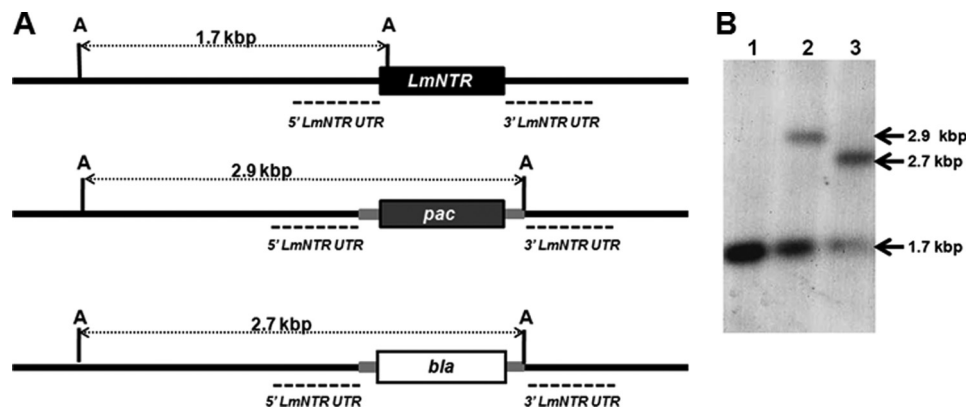
The apparent  $V_{\text{max}}$  and  $K_m$  values of His-tagged LmNTR toward various nitroaromatic and quinone-based substrates were determined in the presence of NADH (“Experimental Procedures”). The  $k_{\text{cat}}/K_m$  value (the “specificity constant”) was then determined providing a useful ratio for comparing the relative rates of LmNTR activity on various substrates.

Compound	Apparent $K_m$ $\mu\text{M}$	Apparent $V_{\text{max}}$ $\text{nmol min}^{-1} \text{mg}^{-1}$	$k_{\text{cat}}/K_m$ $\text{M}^{-1} \text{s}^{-1}$
<b>Nitroimidazole</b>			
Benznidazole	$22.0 \pm 0.4$	$78.7 \pm 1.0$	$2.5 \times 10^3$
Metronidazole	$2.4 \pm 0.0$	$17.9 \pm 0.0$	$5.2 \times 10^3$
<b>Nitrofurans</b>			
Nifurtimox	$9.7 \pm 1.1$	$50.9 \pm 7.0$	$3.7 \times 10^3$
Nitrofurazone	$4.7 \pm 0.6$	$68.3 \pm 10.0$	$1.0 \times 10^4$
Nitrofurantoin	$3.9 \pm 0.5$	$56.7 \pm 8.0$	$1.0 \times 10^4$
<b>Nitrobenzyl</b>			
CB1954	$13.1 \pm 0.1$	$85.9 \pm 1.0$	$4.6 \times 10^3$
LH32	$28.8 \pm 2.7$	$100.7 \pm 10.0$	$2.5 \times 10^3$
LH33	$8.2 \pm 1.0$	$114.2 \pm 15.0$	$9.8 \times 10^3$
LH37	$7.1 \pm 0.4$	$71.7 \pm 5.0$	$7.1 \times 10^3$
<b>Quinone</b>			
Duroquinone	$9.8 \pm 0.3$	$140.6 \pm 5.0$	$1.0 \times 10^4$
Coenzyme Q1	$5.1 \pm 0.4$	$145.2 \pm 11.0$	$2.0 \times 10^4$

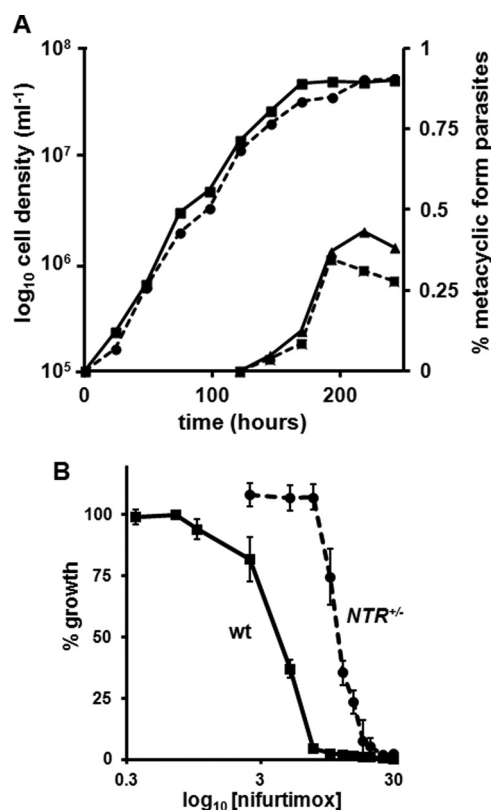
activity of 110 nmol of NADH oxidized per  $\text{min}^{-1} \text{mg}^{-1}$  as compared with 50 nmol of NADPH oxidized per  $\text{min}^{-1} \text{mg}^{-1}$ . Using benznidazole (100  $\mu\text{M}$ ) as substrate, His-tagged LmNTR had an apparent  $K_m$  for NADH of 76  $\mu\text{M}$ , similar to the values reported for *T. brucei* type I nitroreductase (TbNTR) and TcNTR (71 and 86  $\mu\text{M}$ , respectively) (42).

To examine how recombinant LmNTR interacts with NADH, assays were carried out using various concentrations of reductant against a fixed concentration of benznidazole (Fig. 2B). For this substrate, double-reciprocal plots were linear at all concentrations of electron acceptor, with the slopes remaining parallel. When the converse experiments were performed using a fixed concentration of NADH and various amounts of benznidazole, a similar pattern of parallel slopes was observed (Fig. 2B). These patterns are characteristic of a ping-pong mechanism of kinetics, typical for oxidoreductase cascades. However, when benznidazole or NADH levels were above 200  $\mu\text{M}$ , non-competitive substrate inhibition was observed indicating that this mechanism of kinetics only occurs over a limited electron donor/acceptor concentration range (Fig. 2C).

**Functional Analysis of LmNTR in *L. major***—To investigate whether the leishmanial type I NTR can activate nitroaromatic drugs in the parasite itself, we developed *L. major* LmNTR heterozygotes and then examined the susceptibility of the cells to nifurtimox. DNA fragments corresponding to the regions immediately upstream and downstream of *LmNTR* were amplified from genomic DNA and cloned sequentially on either side of cassettes containing puromycin or blasticidin resistance markers. The constructs were linearized, and the purified fragments were transformed into *L. major* promastigotes with clones selected on agar plates containing puromycin or blasticidin. Southern hybridization of genomic DNA showed that one *LmNTR* allele could be disrupted generating heterozygous parasites (*LmNTR* $^{+/-}$ -PAC or *LmNTR* $^{+/-}$ -BLA) (Fig. 3), with no obvious effect on parasite growth or ability to differentiate into infective metacyclic forms (Fig. 4A). To evaluate whether reduction of the *LmNTR* copy number affected resistance to nitroheterocyclic drugs, *LmNTR* $^{+/-}$ -BLA promastigote para-



**FIGURE 3. Disruption of *LmNTR* in *L. major*.** *A*, diagram of the *LmNTR* alleles and the effects of gene disruption. 5'- and 3'-untranslated regions (dashed line) immediately upstream and downstream of *LmNTR* (black box) were amplified and cloned sequentially on either side of a puromycin (*pac*; dark gray box) or blasticidin (*bla*; white box) cassette containing *T. brucei* tubulin intergenic elements required for processing of mRNA (light gray boxes). The position of the predicted Apal (A) sites plus the band sizes (in kbp) obtained after hybridization are shown. *B*, autoradiographs of Apal-digested genomic DNA from *L. major* (lane 1), *LmNTR*<sup>+/+</sup>-PAC and *LmNTR*<sup>+/+</sup>-BLA heterozygous clones (lanes 2 and 3, respectively). Blots were hybridized with labeled 5'-untranslated region of *LmNTR*. Sizes given are in kbp.



**FIGURE 4. Promastigote growth and metacyclogenesis of the *L. major* *LmNTR* heterozygous line.** *A*, *L. major* wild type (square; solid line) and *LmNTR*<sup>+/+</sup>-BLA heterozygote (circle; dotted line) promastigote parasite growth was monitored until cultures were in the stationary phase of growth (time = 240 h). At 120 h onward, the number of metacyclic form parasites in wild type (triangle; solid line) and *LmNTR*<sup>+/+</sup>-BLA heterozygote (cross; dotted line) promastigote culture was determined following purification by agglutination. The data are expressed as % metacyclic load in the total *L. major* population. All curves shown are derived from a single data set and are representative of experiments performed in triplicate. *B*, dose-response curves of nifurtimox on *L. major* wild type (wt) and *LmNTR*<sup>+/+</sup>-BLA heterozygote (*NTR*<sup>+/+</sup>) promastigotes. Data are means from four experiments  $\pm$  S.D., and the differences in susceptibility were statistically significant ( $p < 0.01$ ), as assessed by Student's *t* test.

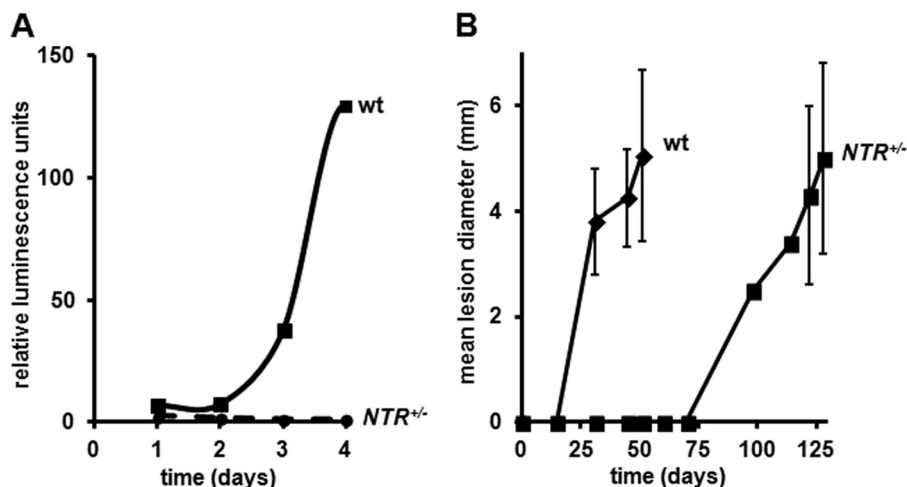
sites were grown in the presence of nifurtimox (Fig. 4*B*). From the resultant dose-response curves, the  $IC_{50}$  value of the heterozygote line was shown to be  $\sim 2$ -fold higher than wild

type parasites; *L. major* wild type cells exhibited an  $IC_{50}$  value of  $6.28 \pm 0.04 \mu M$  as compared with  $11.50 \pm 0.60 \mu M$  for the *LmNTR*<sup>+/+</sup> line.

Attempts (16 independent transformations) to generate *LmNTR* null mutant lines failed, leading us to speculate that the protein is essential to promastigote form parasites. To confirm this, we constructed a range of pTEX- and pIR-SAT1-based episomal and integrative complementation vectors designed to facilitate expression of *LmNTR* or variants tagged at the carboxyl terminus with an epitope derived from the human c-MYC protein. Attempts (20 independent transformations) to generate parasites lines containing an ectopic copy of *LmNTR* failed, leading us to postulate that expression of elevated NTR levels was also deleterious to *L. major* promastigotes. Even when using "amastigote-specific" integrative vectors, where *LmNTR* expression was placed under the control of the well characterized *Leishmania* regulatory genetic element (the amastin 3'-UTR (43)), no recombinant parasites were obtained; the "amastigote-specific" expression system was validated using constructs expressing luciferase where the reporter activity under the control of the amastin 3'-UTR was only 2–10-fold above background, and in the absence of this regulatory genetic element reporter activity was  $>1000$ -fold higher (data not shown). This toxicity may be in part due to the *LmNTR* amino terminus. To facilitate localization, attempts (six independent transformations) to generate *L. major* promastigotes expressing the amino-terminal extension (residues 1–85) of *LmNTR* fused at its carboxyl terminus with GFP or red fluorescent protein failed despite using episomal or integrative expression vectors (data not shown).

To aid the characterization of *L. major* *LmNTR* heterozygotes, *LmNTR*<sup>+/+</sup>-BLA cells were transformed with a DNA fragment that integrates into the *L. major* rRNA promoter/spacer region and facilitates expression of luciferase. Following selection, the luminescence activity of clonal parasite extracts was shown to be  $>10^6$ -fold above background, comparable with lysates generated from luciferase expressing wild type cells. These parasites also exhibit promastigote growth and metacyclogenesis rates equivalent to wild type/non-luciferase-

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**FIGURE 5. Disruption of a single *LmNTR* allele affects infectivity.** *A*, purified *L. major* wild type (*wt*) and *LmNTR*<sup>+/-</sup>-*BLA* heterozygote (*NTR*<sup>+/-</sup>) metacyclic form parasites engineered to express luciferase were used to infect differentiated THP-1 cells. Over a 4-day post-infection period, extracts were generated from each cell line, and the luciferase activity was determined. Following background correction, the luciferase activity was plotted against time. All curves shown are derived from a single data set and are representative of experiments performed in triplicate. *B*, purified *L. major* wild type (*wt*) and *LmNTR*<sup>+/-</sup>-*BLA* heterozygote (*NTR*<sup>+/-</sup>) metacyclic form parasites were inoculated into the rump of BALB/c mice. Periodically, the diameter of the lesion was measured. For each cell line, three mice were infected, and the data are expressed as the mean lesion size (in mm)  $\pm$  S.D. For the heterozygote infections, the lesion sizes on days 98 and 114 are derived from a single mouse as the wounds present on other animals could not be accurately determined.

expressing *LmNTR* heterozygotes (data not shown). Metacyclic form parasites from luciferase-tagged *LmNTR*<sup>+/-</sup> heterozygote and wild type promastigote cultures in the stationary phase of growth were then purified and used to infect differentiated THP-1 macrophage cells. Over the following 4 days, total cell extracts were generated from parasite/mammalian cultures, and the luciferase activity was determined (Fig. 5*A*). After correcting for background luminescence (lysed, uninfected macrophages), the reporter signal generated from tagged wild type amastigotes increased, reaching a plateau by day 4 post-infection. In contrast, *LmNTR*<sup>+/-</sup> heterozygote cultures exhibited luminescence values equivalent to background. This indicates that disruption of one *LmNTR* allele resulted in haploid insufficiency such that *L. major* *LmNTR* heterozygote amastigotes are unable to establish themselves in tissue-cultured mammalian macrophages.

To determine whether the *in vitro* amastigote proliferation defect translates to problems with *in vivo* growth, BALB/c mice were infected with purified wild type or *LmNTR*<sup>+/-</sup> heterozygote *L. major* metacyclic cells. Over a 97-day period, the presence and size of any lesion were monitored (Fig. 5*B*). All three mice infected with wild type parasites developed lesions by day 31 with the lesions gradually increasing in size; all mice were euthanized at day 51. Interestingly, all mice infected with *LmNTR*<sup>+/-</sup> heterozygote cells presented no signs of lesions until day 98, when a single mouse developed a lesion, with the remaining mice developing pathologies 24 days later (day 122). In all cases, the lesion size gradually increased until day 128, when all mice were euthanized. The altered kinetics in pathology development strongly indicate that *LmNTR* function is important in establishing an *L. major* infection *in vivo* with the heterozygote line struggling to grow as amastigote form parasites in BALB/c mice.

*Exploiting the Prodrug Activating Properties of the Leishmania Type I NTR*—We have previously shown that NBPM compounds are effective NTR-activated trypanocidal agents (36).

As an initial screening strategy to determine the potential for exploiting *LmNTR* activity in prodrug activation, we determined whether His-tagged *LmNTR* displayed activity toward a library of NBPMs by monitoring the change in absorbance at 340 nm, corresponding to NADH oxidation (Fig. 6*A*). In total, 22 compounds were screened as follows: eight structures where either a *p*-nitrobenzyl group or an *o*-nitrophenylacetamido group was attached to cyclophosphamide (Table 1) and 14 structures where phosphoramidate mustard is linked to a nitrobenzyl group with various substituents (Table 2). Based on the preliminary biochemical screen, the compounds could be divided into those that were not *LmNTR* substrates (activity  $<20$  nmol of NADH oxidized per  $\text{min}^{-1} \text{mg}^{-1}$ ), those that stimulated a moderate activity (between 20 and 50 nmol of NADH oxidized per  $\text{min}^{-1} \text{mg}^{-1}$ ), and those that were readily metabolized by the parasite enzyme ( $>50$  nmol of NADH oxidized per  $\text{min}^{-1} \text{mg}^{-1}$ ). Using this as a crude measure, three cyclic NBPMs (LH5, -6, and -12) were evaluated as moderate substrates, whereas the remaining five showed little/no activity. In contrast, most of the acyclic structures were metabolized by recombinant *LmNTR* with six (LH27, 31–34, and 37) showing high levels of activity. Of those substrates generating the higher activities, five contained at least one halogen linked to the nitro-substituted benzene ring.

To determine whether there was a correlation between biochemical activity and parasite killing, all NBPMs were initially screened for leishmanicidal activity against *L. major* promastigote and amastigote forms. Out of the 22 compounds, 15 were shown not to affect growth of the insect stage pathogen at 30  $\mu\text{M}$  with all except one (LH19) also having no effect on amastigote growth at a concentration of 10  $\mu\text{M}$  (Table 4). For the remaining leishmanicidal compounds, growth inhibition assays were performed to determine their  $\text{IC}_{50}$  values (Table 4). Many of these displayed appreciable leishmanicidal properties, with five NBPMs (LH31–34 and -37) having significant activity ( $\text{IC}_{50}$  values of  $<10 \mu\text{M}$ ) against both parasite forms. These five com-

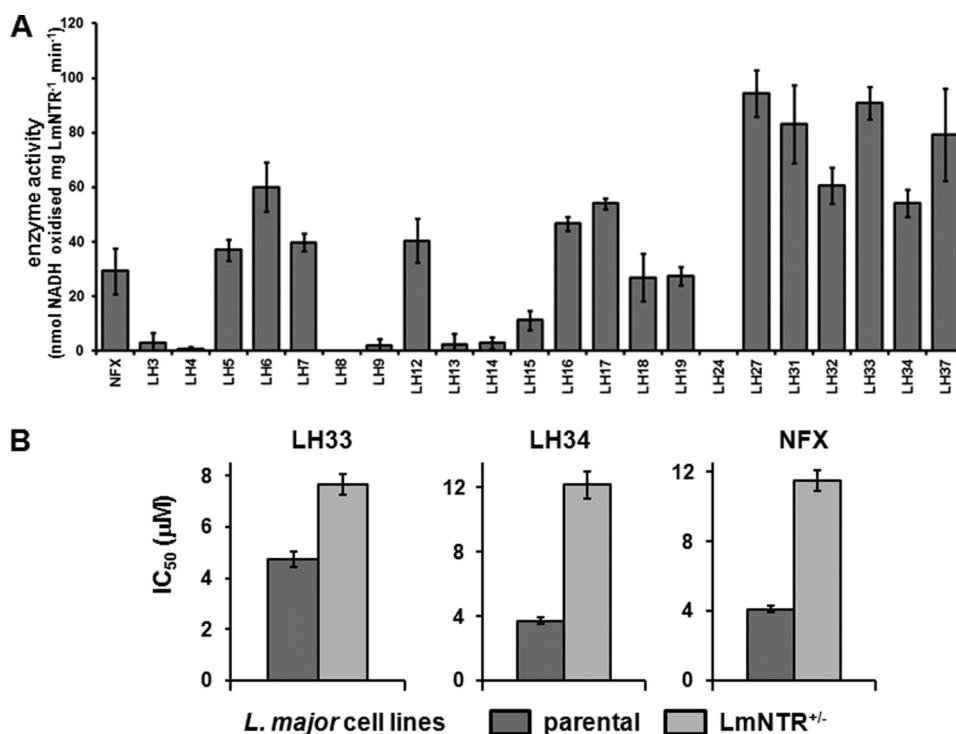


FIGURE 6. **Evaluating nitrobenzylphosphoramidate mustards as LmNTR substrates.** *A*, activity of purified His-tagged LmNTR was assessed by using various NBPMs (100  $\mu\text{M}$ ) as substrate at a fixed concentration of NADH (100  $\mu\text{M}$ ). The values shown are the means of data from three experiments  $\pm$  S.D. LmNTR activity was deemed to be high if it was  $>50$  nmol of NADH oxidized per  $\text{min}^{-1} \text{mg}^{-1}$  (dotted line). The activity obtained when using nifurtimox (NFX) as a substrate is also shown. *B*, susceptibility of *L. major* wild type (wt) and *LmNTR*<sup>+/-</sup>BLA heterozygote (*NTR*<sup>+/-</sup>) promastigotes to LH33 and LH34. Data are means from four experiments  $\pm$  S.D.

**TABLE 4**  
Susceptibility of *L. major* and differentiated THP-1 cells to nitrobenzyl phosphoramidate mustards

Compounds	<i>L. major</i> IC <sub>50</sub>		Differentiated THP-1 IC <sub>50</sub> <sup>b</sup>	Selective toxicity <sup>c</sup>
	Promastigotes <sup>a</sup>	Amastigotes <sup>b</sup>		
	$\mu\text{M}$		$\mu\text{M}$	
Nifurtimox	6.28 $\pm$ 0.04	2.15 $\pm$ 0.01	>100.00	>47
LH3-9, LH12-15, LH17-18, LH24	>30.00	>10.00	ND <sup>d</sup>	ND
LH16	15.60 $\pm$ 1.13	2.75 $\pm$ 0.40	>100.00	>36
LH19	>30.00	4.72 $\pm$ 0.55	>100.00	>21
LH27	16.23 $\pm$ 0.29	4.65 $\pm$ 0.15	>100.00	>22
LH31	9.05 $\pm$ 0.56	7.00 $\pm$ 0.40	>100.00	>14
LH32	4.72 $\pm$ 0.23	1.09 $\pm$ 0.21	>100.00	>92
LH33	5.88 $\pm$ 0.28	0.21 $\pm$ 0.07	>100.00	>476
LH34	3.10 $\pm$ 0.28	0.77 $\pm$ 0.12	>100.00	>143
LH37	1.29 $\pm$ 0.08	2.17 $\pm$ 0.27	>100.00	>46

<sup>a</sup> Data are means from four experiments  $\pm$  S.D.

<sup>b</sup> Data are means from three experiments  $\pm$  S.D.

<sup>c</sup> The therapeutic index of a compound was calculated as a ratio of the IC<sub>50</sub> value against differentiated THP-1 cells to the IC<sub>50</sub> value against amastigote parasites.

<sup>d</sup> ND means not determined.

pounds correspond to structures previously designated “good” LmNTR substrates (Fig. 6).

The eight compounds identified as having appreciable leishmanial activity were assayed for cytotoxicity against differentiated THP-1 cells from which the selective index (IC<sub>50</sub> against the mammalian line/IC<sub>50</sub> against the amastigote parasite) was then determined (Table 4). In all cases, no toxicity to the mammalian line was observed at concentrations up to 100  $\mu\text{M}$ . For three of the halogenated compounds identified as being preferred LmNTR substrates and having anti-parasitic activity against both *L. major* forms (LH32-34), selective index values against amastigote parasites of  $>100$  were observed.

To demonstrate that NTR plays a role in NBPM prodrug activation within the parasite itself, the susceptibility of *L. major* *LmNTR*<sup>+/-</sup>BLA heterozygote promastigotes to LH33 and LH34 was investigated (Fig. 6B). For both compounds, cells with reduced levels of the nitroreductase were up to 3-fold more resistant to the agent than wild type controls; *L. major* wild type cells exhibited IC<sub>50</sub> values of 4.73  $\pm$  0.29 and 3.70  $\pm$  0.40  $\mu\text{M}$  toward LH33 and LH34, respectively, and *LmNTR*<sup>+/-</sup> heterozygote cells displayed values of 7.66  $\pm$  0.23 and 12.14  $\pm$  0.84  $\mu\text{M}$  against these two compounds.

Based on our findings, NBPMs that contain the phosphoramidate mustard as part of an acyclic structure and have halogen substituents on the nitrobenzyl ring are the most readily metabolized by the LmNTR enzyme and represent the most potent agents against both *L. major* promastigotes and amastigotes. Some (LH32, -33, and -34) have IC<sub>50</sub> values of 1  $\mu\text{M}$  or less against the intracellular stage without inducing mammalian cell toxicity.

## DISCUSSION

Activation of most antimicrobial nitroaromatic prodrugs occurs through reactions catalyzed by type I NTRs (24, 26). This group of enzymes was believed to be restricted to bacteria, but it is now apparent that they are also expressed by several “lower” eukaryotes, including fungi and protozoan parasites (20, 26-28). Interestingly, type I NTRs appear to be absent from “higher” eukaryotes, including humans, and it is believed that this difference in the enzymes’ distribution underlies the antimicrobial selectivity of many nitroaromatic prodrugs. Here, we demonstrate that *L. major* expresses a type I NTR that



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possesses many biochemical characteristics displayed by its bacterial counterparts. Functional studies revealed that this activity is essential for the growth of parasite forms found in the insect vector and extremely important to the intracellular forms found in mammalian cells, indicating that this prodrug-activating enzyme is a drug target in its own right.

The activity of all type I NTRs, irrespective of their origin, is dependent on FMN with the enzyme/flavin interaction occurring via a noncovalent linkage, features shared by LmNTR (Fig. 1). His-tagged LmNTR activity was associated with yellow-colored fractions, and boiling released the associated cofactor from the protein backbone with clarified supernatants exhibiting fluorescence profiles under acidic and neutral pH values identical to that observed with FMN (Fig. 1). For the leishmanial enzyme, the cofactor acts as an intermediary, accepting reducing equivalents derived preferentially from NADH and then donating these to a range of nitroaromatic and quinone-based substrates (Table 3). While catalyzing this transfer, His-tagged LmNTR displays a ping-pong type of kinetics, typical of oxidoreductases (Fig. 2). In TcNTR, cofactor binding is mediated by a specific arginine residue at position 90 present in a conserved motif. This amino acid (Arg-96 in LmNTR) plus the adjacent region is present in NTR from *L. major*. Attempts to express a mutated version of the leishmanial enzyme (R96A) failed to generate soluble protein suggesting that the noncovalent FMN binding to the protein backbone may be important in the correct folding of LmNTR.

Based on its substrate preferences (Table 3) and subcellular localization, as inferred from its sequence, LmNTR may function as a mitochondrial NADH:quinone oxidoreductase. In the mitochondria of most eukaryotes, oxidation of NADH to  $\text{NAD}^+$  is normally mediated by complex I of the electron transport chain. This activity serves to translocate protons across the organelle's inner membrane with the concomitant reduction of ubiquinone to ubiquinol. Ubiquinol then drives the cytochrome-dependent respiratory chains that help to form the proton motive force, which ultimately leads to ATP synthesis. In contrast to bloodstream-dwelling trypanosomes, all replicative *Leishmania* forms express functional cytochrome-dependent electron transport chains suggesting that energy production occurs via the well documented route within these parasites (44–46). However, the role played by the *Leishmania* complex I in driving these cascades is unclear. Inhibitor studies indicate that these protozoa possess an atypical complex I with bioinformatic studies revealing a subunit composition distinct from that found in other eukaryotic organisms (44, 47). As LmNTR appears to fulfill an analogous role to the leishmanial complex I, with both functioning as NADH:quinone oxidoreductases, these may act in concert to help maintain the NADH/ $\text{NAD}^+$  balance within the parasite mitochondrion.

The endogenous function of LmNTR is essential to replicating *L. major*: LmNTR could not be deleted from noninfectious promastigote parasites with the heterozygotes unable to establish an infection in cultured macrophages and struggled to generate lesion pathology in mice (Fig. 5). This is similar to the situation in *L. donovani* but distinct from that observed in trypanosomes where the essential nature of NTR is only apparent in the replicating forms present in the mammalian host (26,

48). As we were unable to express elevated levels of LmNTR in *L. major* heterozygotes and wild type promastigotes, the appropriate complementation experiments could not be performed. Intriguingly, complementation studies could be conducted using *L. donovani* promastigotes (48); *L. donovani* NTR null mutants could be generated only in the presence of an ectopic copy of *L. major* NTR. The deleterious effect of expressing LmNTR at elevated levels may not be due to the enzymatic activity of the protein itself but could reflect an inherent problem associated with the amino-terminal extension. Therefore, when expressed at levels higher than that found in wild type (and heterozygote), the LmNTR extension may interfere with mitochondrial transporter function or could interact with other essential cellular components thereby inhibiting their activities.

Nitrobenzylphosphoramidate mustards are a novel class of compounds incorporating chemical motifs present in several DNA alkylating anti-cancer agents (30–33). They contain a nitrogen mustard moiety coupled to a nitrobenzyl ring via a phosphoramidate linker and are designed to function as prodrugs specifically tailored to undergo activation in reactions catalyzed by type I NTRs. Here, we performed a structure-activity relationship on a library of NBPMs employing biochemical and leishmanicidal screens (Fig. 6 and Table 4). When using a group of NBPMs where the phosphoramidate linker was part of a cyclic structure (Table 1), recombinant LmNTR protein was shown to metabolize some (3 out of 8) at reasonable rates, but this failed to translate into an anti-parasitic activity. Screens involving NBPMs, where the phosphoramidate was part of a linearized linker (Table 2), yielded similar results for 6 out of 14 compounds with the remaining NBPMs being effective LmNTR substrates and displaying leishmanicidal effects. When comparing the anti-parasitic properties exhibited by the eight most potent NBPMs against the two replicative *L. major* life cycle stages, intracellular amastigote parasites were generally more susceptible to the agent under study than the promastigote form; LH33 had  $\text{IC}_{50}$  values of  $5.88 \pm 0.28$  and  $0.21 \pm 0.07 \mu\text{M}$  against promastigote and amastigote form parasites, respectively, whereas LH34 exhibited values of  $3.10 \pm 0.28$  and  $0.77 \pm 0.12 \mu\text{M}$ . To conclusively demonstrate that the LmNTR plays a role in NBPM activation in the parasite itself, the susceptibility of *L. major* LmNTR<sup>+/-</sup> heterozygous cells to LH33 and LH34 was evaluated. In both cases, reduction of the LmNTR gene copy number resulted in a resistance phenotype in agreement with observations made using recombinant trypanosomes and *L. donovani* toward other nitroaromatics (Fig. 6) (26, 48). As mammalian cells lack a type I NTR activity, they should be less susceptible to prodrugs that rely on this mechanism of activation. When the eight most potent leishmanicidal NBPMs were screened against the macrophage line in which the intracellular parasites were cultured, no cytotoxicity was observed at values up to  $100 \mu\text{M}$  (Table 4). In terms of relative toxicity values, the two agents showing the highest potency against amastigote cells were >476 (LH33) and >143 (LH34) more toxic to the parasite than this particular mammalian line.

We have now shown that *L. major* expresses a type I NTR that in the related trypanosomal parasites activates the clinically used prodrugs nifurtimox and benznidazole (26, 49, 50).

The leishmanial enzyme displays characteristics typical of this group of oxidoreductases being able to noncovalently bind with FMN and, through a ping-pong mechanism, utilizes NADH to reduce a wide range of nitroaromatics and quinones. We exploited this activity and identified several leishmanicidal NBPM compounds that have little/no cytotoxicity in mammalian cells. Based on substrate preference, the *L. major* type I NTRs can be regarded as NADH:quinone oxidoreductase, with this activity being important to the replicative parasite forms found in the insect and mammalian host. Therefore, our data indicate that the leishmanial enzyme can be used in drug development in two ways: either as an activator of new nitroaromatic/quinone-based prodrugs and/or through the use of inhibitors targeting this essential activity. Additionally, these two approaches could be complementary; an *L. major* strain showing resistance to drugs developed to inhibit LmNTR could theoretically be sensitive or even hypersensitive to prodrugs activated by this leishmanial enzyme and vice versa.

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