Dissecting the Metabolic Roles of Pteridine Reductase 1 in Trypanosoma brucei and Leishmania major

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Leishmania parasites are pteridine auxotrophs that use an NADPH-dependent pteridine reductase 1 (PTR1) and NADH-dependent quinonoid dihydropteridine reductase (QDPR) to salvage and maintain intracellular pools of tetrahydrobiopterin (H4B). However, the African trypanosomosome lacks a credible candidate QDPR in its genome despite maintaining apparent QDPR activity. Here we provide evidence that the NADH-dependent activity previously reported by others is an assay artifact. Using an HPLC-based enzyme assay, we demonstrate that there is an NADPH-dependent QDPR activity associated with both TbPTR1 and LmPTR1. The kinetic properties of recombinant PTR1s are reported at physiological pH and ionic strength and compared with LmQDPR. Specificity constants (kcat/Km) for LmPTR1 are similar with dihydrobiopterin (H2B) and quinonoid dihydrobipterin (qH2B) as substrates and about 20-fold lower than LmQDPR with qH2B. In contrast, TbPTR1 shows a 10-fold higher kcat/Km for H2B over qH2B. Analysis of Trypanosoma brucei isolated from infected rats revealed that H4B (430 nM, 98% of total biopterin) was the predominant intracellular pterin, consistent with a dual role in the salvage and regeneration of H2B. Gene knock-out experiments confirmed this: PTR1-nulls could only be obtained from lines overexpressing LmQDPR with H2B as a medium supplement. These cells grew normally with H2B, which spontaneously oxidizes to qH2B, but were unable to survive in the absence of pterin or with either biopirin or H2B in the medium. These findings establish that PTR1 has an essential and dual role in pterin metabolism in African trypanosomes and underline its potential as a drug target.

Tetrahydrobiopterin (H4B)2 is a biologically significant pterin that functions as an essential cofactor for several enzymes. In mammalian cells these enzymes catalyze reactions such as the hydroxylation of aromatic amino acids (phenylalanine, tyrosine, and tryptophan) by their respective hydroxylases (1–3), as well as the hydroxylation and cleavage of glyceryl ethers by glyceryl-ether monooxygenase (4, 5). In addition, H4B is also required for the coupling of all three isoforms of nitric-oxide synthases (NOS) for the production of nitric oxide (NO), both an important signaling molecule as well as a powerful antimicrobial agent (6–8). In mammalian cells, H2B is synthesized de novo from guanosine triphosphate (GTP) and oxidized to H2B-4a-carbinolamine following its utilization in hydroxylation reactions (9–11). The oxidized pterin is subsequently regenerated to its fully reduced form via the quinonoid dihydrobipterin (qH2B) intermediate by the enzymes pterin 4a-carbinolamine dehydratase (PCD; EC 4.2.1.96) and quinonoid dihydrobiopterin reductase (QDPR; 6,7-dihydropteridine reductase, EC 1.5.1.34) (12–14). qH2B can also be non-enzymatically generated by the spontaneous oxidation of H2B before undergoing further rearrangement to form 7,8-dihydrobipterin (H1B), a process accelerated by the presence pro-oxidants (15–17). H1B can then be reduced to H2B by mammalian dihydrofolate reductase (DHFR), a key enzyme in folate metabolism, in an alternative salvage reaction (18).

Protozoan parasites in the family Trypanosomatidae are the causative agents of several neglected tropical diseases, including the leishmaniasises, caused by the Leishmania spp. and the African trypanosomiasises caused by Trypanosoma brucei ssp. The current understanding of pterin metabolism in trypanosomatids stems largely from studies on Leishmania spp. and also the insect trypanosomatid, Crithidia fasciculata. These parasites lack the biosynthetic pathway to H4B and are auxotrophic for pterins (19–21). In Leishmania, salvage of extracellular biopirin involves uptake predominantly through the biopirin transporter 1 (BT1) and the fully oxidized pterin is then reduced sequentially to its dihydro- and tetrahydro-forms by the NADPH-dependent enzyme pteridine reductase 1 (PTR1; EC 1.5.1.33) (22, 23). The end product of this salvage pathway (H2B) is essential for growth of the promastigote stage of Leishmania major, because PTR1-null mutants are unable to grow in culture unless supplemented with H2B or H1B (23).

The precise functions of H4B within Leishmania spp. are not fully understood. H4B deficiency in the insect vector (promastigate) stage of the life cycle promotes increased differentiation into the mammalian-infectious metacyclic promastigate form (24), but the underlying mechanism remains obscure. Unlike mammalian cells, glyceryl-ether monooxygenase in Leishmania donovani is dependent upon NADPH instead of H2B (25). With the exception of phenylalanine hydroxylase (PAH), genes for NOS and other aromatic amino acid hydroxylases are not annotated in any Leishmania genome. Moreover, LmPAH appear to be non-essential for the growth of L. major in vitro (26) indicating that there must be other crucial pterin-depen-
dent processes within these parasites. Whatever the role(s) of H4B within these parasites may be, L. major also harbor a putative PCD and a well-characterized QDPR (27) suggesting they are able to regenerate H4B in a fashion not unlike mammalian cells. A role for H4B in defense against oxidative stress in Leishmania has also been suggested by two independent studies, where PTR1-null mutants of L. major showed increased susceptibility to oxidative stress (28, 29). However, the underlying mechanism remains unclear.

In contrast to the leishmania parasites, very little is known about pterin metabolism in T. brucei. However, it would appear to be considerably different. Knockdown of PTR1 levels in T. brucei by RNA interference is lethal in vitro and cannot be rescued by supplementation with either H4B or H2B (30), unlike L. major PTR1-null mutants (24). In addition, PTR1 knockdown abolishes infectivity of T. brucei to mice (30), unlike L. major ptr1−/− that retains infectivity to mice (24). Finally, despite a report of QDPR activity in T. brucei (27), no obvious candidate genes for QDPR (or PAH) have been identified in its genome. The current study provides an explanation for some of these anomalies and provides convincing evidence that PTR1 is an essential drug target in the African trypanosome, T. brucei.

**EXPERIMENTAL PROCEDURES**

**Organisms and Reagents**—All pterins were purchased from Schircks laboratories. Other chemicals and reagents used in this study were of the highest grade and purity available. T. brucei procyclic-form strain 29–13 (31) was cultured at 28 °C in SDM-79 medium (32) supplemented with 50 μg ml−1 hygromycin (Roche) and 15 μg ml−1 gancyclovir sulfate (G418, Invitrogen). T. brucei bloodstream-form “single marker” S427 was cultured at 37 °C in either HMI-9T medium (33) or low folate medium (34), with both media supplemented with 15 μg ml−1 of G418. Alternatively, parasites were purified from infected rat blood (35). Additional H4B (1 μM) was included in cultures of T. brucei cells expressing LmQDPR.

**Analysis of Pterins by HPLC**—Reverse-phase HPLC was carried out on an ion-paired Ultrasphere C18 column (HPLC Technology) using a Dionex UltiMate 3000 system coupled to a Dionex RF-2000 fluorometer. Pterins were separated and eluted using an isocratic mobile phase of 20 mM (Na+) phosphate pH 6.5, 4% (v/v) methanol at a flow rate of 1 ml min−1. Prior to HPLC analysis, biological samples were oxidized with iodine in the presence of 0.1 M HCl or NaOH (36, 37). Precipitated proteins were removed by centrifugation (20,000 × g, 4 °C, 20 min) and supernatants analyzed by HPLC. Oxidized pterins were detected fluorometrically using excitation and emission wavelengths of 360 and 440 nm, respectively. Biop- terin plus H4B was quantified by determining the total biop- terin in alkaline-oxidized samples. Concentrations of H4B were calculated by subtracting total biop- terin in alkaline-oxidized samples from total biop- terin in acid-oxidized samples. Pterin standards were quantified using published extinction coefficients (38).

**Pterin Content in T. brucei and Mouse Serum**—Parasites were harvested by centrifugation (800 × g, 4 °C, 10 min) and washed once with phosphate buffered saline (PBS, Invitrogen). Cell pellets were resuspended in PBS and aliquots (100 μl) were oxidized, deproteinized, and analyzed as above.

The concentration of pterins in serum from infected and uninfected mice were determined by infecting 25 Balb/c mice with 1 × 107 bloodstream T. brucei parasites via intra-peritoneal injection. Parasitaemia was evaluated as previously described (34). Groups of 5 mice were exsanguinated under anesthesia on day zero and daily thereafter until a lethal parasitaemia was achieved on day 4. Blood samples were allowed to clot, centrifuged (1,000 × g, 4 °C, 10 min) and aliquots of supernatant (100 μl) were immediately oxidized, deproteinized, and analyzed by HPLC.

**Enzyme Kinetics—LmQDPR**—LmQDPR was cloned into the pET15b vector. Histidine-tagged recombinant TbPTR1, LmPTR1, and LmQDPR were expressed and purified as published (27, 39, 40). LmPTR1 and LmQDPR were further purified to homogeneity by anion-exchange chromatography (39). PTR1 activity was measured by HPLC using H2B as a substrate (37) and spectrophotometrically on a UV-1601 spectrophotometer (Shimadzu) using the quinonoid substrates qH4B or qDMH2P generated from the corresponding tetrahydropterins using horseradish peroxidase (HRP) and H2O2 (27).

Rates of oxidation of NADPH or NADH at 340 nm were calculated using the extinction coefficient 6.22 mM cm−1. Initial linear rates were measured over 120 s and corrected for nonspecific oxidation of NADH or NADPH. One unit of enzyme activity is defined as one μmol of substrate used (spectrophotometric assays) or product formed (HPLC) per min.

The pH optimum of TbPTR1 for H2B was determined using overlapping buffers of sodium citrate (pH 2.6 to 5.5) and HEPES/MES/CHES (pH 5.5–10), adjusted to a constant ionic strength of 150 mM with NaCl. All subsequent enzymatic assays were carried out in 50 mM HEPES buffer, adjusted to pH 7.4 and an ionic strength of 100 mM using KOH and KCl. All assay mixtures were pre-equilibrated to 25 °C before the reaction was initiated by the addition of the appropriate pterin substrate.

The Kapp of TbPTR1 for NADPH was determined using a fixed concentration of the enzyme (1.1 nM) and H4B (25 nM) in the presence of varying concentrations of NADPH (0.2–200 μM). Using 1.1 nM enzyme and 100 μM NADPH cofactor, the concentration of H4B was varied from 10 to 3000 nM to determine the Kapp of TbPTR1 for H4B. For LmPTR1, the concentration of enzyme was increased to 5 nM and H4B concentration varied from 50 to 4000 nM. The Kapp of TbPTR1 and LmPTR1 with the quinonoid dihydropterin substrates were determined in the same buffer by varying the concentrations of tetrahydropterins from 0.8 to 100 μM in the presence of 85 nM enzyme, 50 μM NADPH, 20 μg ml−1 HRP, and 0.9 mM H2O2. These results were fitted by non-linear regression to Equation 1 for high substrate inhibition in GraFit.

\[
\nu = \frac{V_{\text{max}}}{1 + \frac{K_m}{S} + \frac{S}{K_i}}
\]

(Eq. 1)

The Kapp of LmQDPR for the quinonoid substrates were measured in a similar fashion, using 2 nM enzyme and 50 μM NADH. Data were fitted to the Michaelis-Menten equation.
Knock-out of PTR1 in Bloodstream Form T. brucei—Knock-out constructs of TbPTR1 were prepared as previously described (30). To generate the tetracycline-inducible LmQDPR_pLew82 overexpression construct, the open reading frame of the gene was amplified from the LmQDPR_pET15b construct using primers 5’-ggcaggccaggttacgtaaaatgtaac-3’ and 5’-gcggagatgctcttaaaagttgccga-3’ (restriction enzymes sites HindIII and BamHI highlighted in bold italics) and cloned into the pLew82 vector (31). LmQDPR_pLew82 was transfected into wild-type (WT) bloodstream form T. brucei by electroporation for targeted integration into the ribosomal DNA locus (31) and parasites overexpressing LmQDPR (‘‘WT) selected for resistance to phleomycin (5.0 μg ml⁻¹, InvivoGen). A cloned cell line of ‘‘WT was generated by limiting dilution and used for subsequent studies. Single (‘‘SKO) and double knock-out lines (‘‘DKO) of TbPTR1 were generated using puromycin and hygromycin selection (30).

Southern Analysis—Genomic DNA (5 μg) was prepared and digested with SacI and SpH1 for Southern analysis (30). Probes of the open reading frames of TbPTR1 and LmQDPR were prepared using the PCR DIG Probe Synthesis Kit (Roche). The blot was sequentially probed and processed using the DIG Detection kit (Roche), according to the manufacturer’s instructions.

Semi-quantitative RT-PCR—Levels of TbPTR1 and LmQDPR mRNA in WT and transfected parasites were determined by semi-quantitative RT-PCR analysis using the One Step RT-PCR Kit (Qiagen), according to the manufacturer’s instructions. RNA was prepared from log-phase (1 × 10⁶ cells ml⁻¹) cultures of bloodstream trypanosomes using RNAeasy mini prep kit (Qiagen). DNA was removed from samples using the DNAfree kit (Ambion) and RNA was quantified at 260/280 nm using a Biowave II spectrophotometer (VWP). Oligonucleotide sequences of TbPTR1 (5’-TGTACGTGCAATCTTT-3’ and 5’-AAGCAGTTGACC-3’) and LmQDPR (5’-GCTGAGACAATGGTCTTT-3’ and 5’-TGAGAGAAGGTCTCATT-3’) were designed using the Beacon Design software (PREMIER Biosoft International) to generate products of 102 and 83 bp, respectively. RNA (0.5 μg) from each cell type was reverse transcribed using the following conditions: 50 °C × 30 min and 95 °C × 15 min for 1 cycle; followed by 95 °C × 1 min, 60 °C × 1 min and 72 °C × 1 min for 30 cycles; and a final extension at 72 °C × 10 min for 1 cycle. PCR products were analyzed by agarose gel-electrophoresis.

Preparation of Cell Lysates for Enzymatic Studies—Log-phase cultures of T. brucei (1 × 10⁷ cells ml⁻¹ for procyclics; 1 × 10⁶ cells ml⁻¹ for bloodstream) were harvested by centrifugation (800 × g, 10 min, 4 °C) and washed twice with PBS. Clarified lysates of parasites were prepared as published (33). Trypanothione reductase activity in lysates was determined to ensure adequate extraction of parasites (41). Specific activities of PTR1 and qDPR were determined using 25 nm H₂B plus 100 μM NADPH or 4 μM H₂B or 10 μM DMH₃P plus 100 μM NADH as substrates, respectively.

RESULTS

Intracellular Pterin Analysis of T. brucei—Initial studies established that pterin standards can be optimally separated and quantified on a C₁₈ column using an isocratic mobile phase containing 20 mm (Na⁺) phosphate and 4% methanol (Fig. 1A). Biopterin is the major intracellular pterin in bloodstream form T. brucei harvested from infected rats (Fig. 1B). Total biopterin content is proportional to cell number between 1 × 10⁷ and 1.2 × 10⁸ per assay (Fig. 1C), yielding a total biopterin content of 2.57 ± 0.03 pmol per 10⁸ cells. This is equivalent to 440 nm, based on a cell volume of 5.8 μl (42). Differential oxidation under acidic or alkaline conditions indicated that H₂B plus qH₂B constituted more than 96% (430 nm) of the total intracellular biopterin. The fluorescent peak which co-elutes with pteridoxamine (2-amino-1H-pteridin-4-one or “pterin”) (Fig. 1B), is likely to be derived from tetrahydrofolate, following oxidation by iodine under acidic conditions (36).
Pterin Content of Serum from T. brucei-infected Mice—In previous studies, elevated levels of NOS activity and NO have been observed in T. brucei-infected animals (43). H4Bi is required for the production of NO and circulating levels of H4Bi can be elevated as a mechanism of immune system activation (44, 45). To examine if infection with T. brucei stimulates H4Bi production in vivo, biopterin concentrations were determined during the course of the infection (Fig. 2). In keeping with previous studies (36, 38), the basal level of total biopterin in uninfected mouse serum was determined to be 128 ± 100 nM of which 57% was recovered as H4B. As the infection progressed, total biopterin in serum gradually increased reaching 292 ± 8 (n = 5) nM by day 4 when parasitaemia was at its peak (~1 × 10^9 ml^-1). The levels of biopterin plus H2B remained relatively constant throughout the study, whereas H4B increased ~3-fold from 73 ± 14 nM on day 0 to 230 ± 6 on day 4. Thus, infection with T. brucei does indeed stimulate H4B production in the host.

Kinetic Studies on TbPTR1—PTR1 is pivotal for salvage of biopterin in trypanosomatids, with its essentiality in T. brucei previously established by RNA interference studies (30). Kinetic parameters of TbPTR1 for the reduction of H2B vary considerably (37, 39), depending upon the pH optimum (3.7 versus 6.0), enzyme concentration (4.8 versus 700 nM) and the assay format (direct spectrophotometric versus cytochrome c-coupled method). Because PTR1 has been determined to be a cytosolic enzyme in T. brucei (30), the pH optimum of the enzyme was re-examined using H2B as substrate. Using the HPLC-based assay, the enzyme displays a bell-shaped pH profile with a broad pH optimum from 5.5 to 8 for H2B (Fig. 3A).

Because the reported cytosolic pH of T. brucei is 7.4 (46), recombinant TbPTR1 was subsequently characterized at this physiologically relevant pH and ionic strength. As previously observed under different assay conditions (37, 39), H2B was found to inhibit TbPTR1 at high substrate concentrations, yielding a K_m^app = 29.4 nM, K_i = 1.2 μM, and k_cat/K_m = 1.7 × 10^6 M^-1 s^-1 (Fig. 3B; Table 1). The K_m...
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Summary and comparison of the kinetic characterization of TbPTR1, LmPTR1, and LmQDPR with various substrates

| Units | TbPTR1 | LmPTR1 | LmQDPR |
|-------|--------|--------|--------|
| | $K_m^{app}$ | $K_i$ | $k_{cat}/K_m^{app}$ | | $K_m^{app}$ | $K_i$ | $k_{cat}/K_m^{app}$ | | $K_m^{app}$ | $K_i$ | $k_{cat}/K_m^{app}$ |
| | $\mu M$ | | $M^{-1} s^{-1}$ | | $\mu M$ | | $M^{-1} s^{-1}$ | | $\mu M$ | | $M^{-1} s^{-1}$ |
| $H_2B$ | 0.0294 ± 0.0061 | 1.16 ± 0.27 | 0.05 ± 0.02 | | 3.32 ± 0.48 | 0.15 ± 0.022 | 0.57 | | 4.12 ± 0.44 | 0.38 |
| $qH_2B$ | 3.36 ± 0.70 | 3.14 ± 0.70 | 0.54 | | 16.6 ± 1.9 | 1.22 ± 0.29 | 1.84 | | 21.0 ± 4.3 | 92.2 ± 18.9 |
| $qDMH_P$ | 4.3 ± 1.0 | 0.43 | | | 0.26 ± 0.026 | 0.21 ± 0.02 | | 0.44 | 0.44 |

$*P$ Not applicable.

* Determined by HPLC method with 25 nM $H_2B$ and 100 nM NADPH.
$^a$ Determined by spectrophotometric method with 4 nM $qH_2B$ and 100 nM NADPH.

NADPH, unlike QDPR which has a strong preference for NADH (27). As observed with $H_2B$, $qH_2B$ also inhibited TbPTR1 at high substrate concentrations (Fig. 4A; Table 1). Substrate inhibition was evident, but less pronounced when the quinonoid form of the $H_2B$ analog 6,7-dimethyltetrahydropterin (DMH$_P$) was used as substrate (Fig. 4D; Table 1). Based on the specificity constants ($k_{cat}/K_m$) in Table 1 TbPTR1 has a substrate preference in the order $H_2B > qH_2B > qDMH_P$.

Unexpectedly, recombinant PTR1 from L. major was also found to be able to reduce both of these quinonoid substrates, with similar specificity for the cofactor NADPH and susceptibility to substrate inhibition. These findings are at odds with previous reports that LmPTR1 is unable to catalyze these reactions (27, 48). The kinetic parameters ($K_m^{app}$, $K_i$, and $k_{cat}/K_m^{app}$) for LmPTR1 were found to be within 3-fold of the values observed for TbPTR1, although LmPTR1 was more susceptible to substrate inhibition by $qDMH_P$ (Fig. 4, B and E; Table 1).

The efficiency of both PTR1 enzymes in reducing quinonoid dihydropterins was directly compared with the authentic and well-characterized QDPR from L. major (27). The kinetic parameters of LmQDPR with qDMH$_P$ as substrate were found to be consistent with previously published values (27). Comparing the two substrates, LmQDPR exhibited a lower $K_m^{app}$ (~5-fold) as well as a higher catalytic efficiency (~4-fold) with its natural substrate, $qH_2B$, than with qDMH$_P$ (Fig. 4, E and F; Table 1). Interestingly, these $k_{cat}/K_m$ values were also found to be very similar to those determined for trypanosomatid PTR1 enzymes. However, inhibition by high $qH_2B$ concentrations was not observed with LmQDPR. Collectively, these results suggest that these enzymes have a higher affinity and a substrate preference for $qH_2B$ over qDMH$_P$. More significantly, the lower $k_{cat}/K_m$ values also suggested that both PTR1 enzymes are less catalytically efficient in reducing quinonoid dihydropterins compared with LmQDPR.

QDPR Activity in T. brucei Extracts—The ability of recombinant TbPTR1 to reduce quinonoid substrates suggested that this enzyme could be responsible for the QDPR activity previously detected in lysates of procyclic-form T. brucei (27).
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However, the QDPR activity in these lysates was apparently NADH-dependent, while, as shown here, TbPTR1 is specific for NADPH as cofactor in its reduction of quinonoid dihydropterins.

Using NADH and qDMH₄P (generated from DMH₄P by HRP and H₂O₂), we were able to confirm the presence of QDPR activity in extracts of L. major promastigotes with a specific activity of 33.1 ± 1.5 mU mg⁻¹, in good agreement with published data (27). An apparent QDPR activity was also present with qDMH₂P in procyclic-form of T. brucei as illustrated by the spectrophotometric traces shown in Fig. 5A. After establishing the baseline rate of oxidation of NADH for 5 min, DMH₂P was added to the reaction mixtures to initiate the reaction. With T. brucei extract (solid blue line) there was an initial rapid decrease in absorbance that returned to the background rate of NADH oxidation as illustrated by the two dotted blue lines. The magnitude of this displacement was proportional to the amount of extract added to the assay (not shown) and the reaction was completely abolished by heat treatment of the enzyme sample (Fig. 5A, gray line). No such activity was observed with (mammalian) bloodstream form extracts (Fig. 5A, green line). Addition of LmQDPR to the procyclic extract led to a complete oxidation of NADH (Fig. 5A, purple line), as would be expected based on the recycling nature of the assay. In contrast to the non-linear behavior of the procyclic extract (Fig. 5B, blue line), oxidation by the L. major enzyme extract was completely linear for the initial 200 s (Fig. 5B, red line) and did not return to baseline levels during the experiment. Moreover, this phenomenon with extracts of T. brucei (insect) procyclic forms appears to be specific to qDMH₄P as substrate, because it was not observed when qH₂B was used as substrate (Fig. 5B, blue line) and the residual rate was zero after correction for the non-enzymatic oxidation of NADH (Fig. 5B, black line). In contrast, L. major extract showed substantial NADH-dependent activity with qH₂B (Fig. 5B, red line) as did recombinant LmQDPR (purple line). These findings demonstrate that DMH₄P is not a suitable substrate for determining QDPR activity in procyclic-form T. brucei.

Although QDPR activity with qH₂B and NADH was observed with L. major extracts, activity could not be detected in lysates of wild-type T. brucei in the presence of NADH, whereas it was readily measurable in T. brucei cell lines overexpressing LmQDPR (Table 2). No PTR1-dependent QDPR-like activity could be detected with qH₂B and NADPH in T. brucei lysates using the spectrophotometric method. However, even though the specific activity of recombinant TbPTR1 for qH₂B is 5-fold higher than H₂B (360 versus 73.6 mU mg⁻¹, respectively) the activity with qH₂B as substrate would still be too low for detection by this method. Unfortunately, our current HPLC method is unable to distinguish between the substrate, qH₂B and the product of the reaction, H₄B.

Knock-out of PTR1 in Bloodstream Form T. brucei—PTR1-null mutants of L. major have been previously shown to be viable provided the culture medium was supplemented with either

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**Figure 4. Comparative studies of the reduction of quinonoid dihydropterins by TbPTR1, LmPTR1, and LmQDPR.** PTR1 activity was determined by HPLC and QDPR activity was measured spectrophotometrically as described under “Experimental Procedures.” A and D, rate behavior of TbPTR1 as a function of varying substrate concentrations for qH₂B and qDMH₄P, respectively, determined in the presence of a fixed (saturating) concentration of NADPH. B and E, rate behavior of LmPTR1 as a function of varying concentrations of qH₂B and qDMH₂P, respectively, determined in a similar fashion. C and F, rate behavior of LmQDPR as a function of varying substrate concentrations of qH₂B and qDMH₂P, respectively, determined in the presence of a fixed (saturating) concentration of NADH. Each data point is the mean ± S.E. (n = 3). The solid lines represent the unweighted non-linear fit to either the substrate inhibition equation (PTR1) or Michaelis-Menten equation (QDPR). Kinetic parameters are reported in Table 1.
When similar attempts to knock-out PTR1 in *T. brucei* were carried out in growth medium supplemented with H$_2$Bo rH$_4$B (or a combination of both), PTR1-null mutants could not be obtained (30). Bearing in mind the instability of H$_4$B in neutral or alkaline solutions, it is likely that H$_4$B was rapidly oxidized to qH$_2$B in culture medium (15–17).

Because PTR1 is likely to be solely responsible for the regeneration of H$_4$B in *T. brucei*, we investigated whether a functional QDPR plus supplementation with H$_4$B could serve as an effective metabolic rescue strategy.

First, bloodstream *T. brucei* was transfected with the tetracycline-inducible *LmQDPR* pLew82 vector construct and trypanosomes selected for stable integration into the ribosomal DNA locus by selection for resistance to phleomycin. The successful generation of a wild-type QDPR overexpressing cell line (oeWT) was verified by Southern blot analysis (Fig. 6A), and by RT-PCR (Fig. 6B). Because of the “leaky” nature of the promoter of pLew82, tetracycline induction was not required for cells to express measurable levels of *LmQDPR* mRNA (Fig. 6B). In contrast to WT *T. brucei*, it was now possible to measure QDPR activity in the bloodstream and procyclic forms of *T. brucei* in the presence of H$_2$Bo rH$_4$B (Fig. 5).
activity in the extracts of WT parasites demonstrating that LmQDPR is functionally active (Table 2). Parasites expressing LmQDPR (doubling time, 8.4 h) were also found to have marginally slower growth rates than WT trypanosomes (doubling time, 6.9 h), suggesting that the QDPR might be toxic to these cells (Fig. 6C). Indeed, increased expression of QDPR by induction with tetracycline was lethal to cells (data not shown).

Having successfully created the WT line, further rounds of transfection were done to delete PTR1. The deletion of one copy of PTR1 (SKO) did not result in any changes in growth (doubling time 8.8 h) compared with the WT cells (Fig. 6C). However, the deletion of the second copy (DKO) resulted in a 1.7-fold decrease in growth rate (generation time 15.3 h; Fig. 6C). The loss of both PTR1 genes and their expression were confirmed by Southern blot analysis and RT-PCR respectively (Fig. 6A and B). DKO parasites were only viable in folate-rich medium supplemented with H4B. While they continued to grow normally for 11 days in the absence of H4B, cell numbers dropped precipitously thereafter, such that no surviving cells were evident by day 16 (Fig. 6C).

Experiments were then carried out to determine whether bipterin or H2B could substitute for H4B in supporting growth of DKO cells in low folate medium (Fig. 6D). In the absence of added pterin, the cells were completely dead by day 4, whereas addition of bipterin or H2B prolonged survival to 14 and 16 days, respectively. Only when medium was supplemented with H4B at each subculture were the trypanosomes able to grow normally. These results confirm the dual role of TbPTR1 in the salvage and regeneration of H4B, cementing its status as a validated drug target for the treatment of HAT.

DISCUSSION

The studies reported here have identified some striking differences in pterin metabolism between T. brucei and L. major. Our findings argue strongly in favor of the absence of a bona fide NADH-dependent QDPR in T. brucei for the following reasons. First, unlike L. major cell extracts, oxidation of NADH by qDMH2P catalyzed by procyclic-form lysates is non-linear and does not go to completion by recycling between the quinonoid and tetrahydro-forms. Second, NADH oxidation is not observed with the physiological substrate, qH2B. Third, only cells overexpressing LmQDPR display readily detectable QDPR activity with NADH as electron donor, indicating that the fail-
ure to detect activity in wild-type cell extracts was not due to artifacts such as endogenous inhibitors. Finally, these observations are entirely consistent with the absence of a credible QDPR orthologue in the *T. brucei* genome.

The exact nature of the reaction observed in procyclic-form *T. brucei* remains to be elucidated. At present we have established that all components of the assay are required, including HRP and H$_2$O$_2$, suggesting that the reaction has a requirement for qDMH$_2$P. The activity is heat sensitive and the amount of NADH oxidized is proportional to the amount of extract, suggesting a heat labile protein or metabolite may be involved. It also appears to be specific to *T. brucei* procyclic-form, because the activity is absent in bloodstream forms.

One important point for consideration is whether the QDPR activity associated with PTR1 is physiologically relevant to *T. brucei*. Our studies indicate that the total bipterin concentration in bloodstream forms is 440 nM and PTR1 is 2.5 nM. However, only 2% (10 nM) is present as bipterin or more likely as H$_2$B, since this is the end product of oxidation of H$_2$B or qH$_2$B (15, 16). Thus, under physiological conditions the enzyme is operating below the $K_{m}$ (29 nM) for H$_2$B. This supports the hypothesis that enzymes evolve to function at substrate concentrations equivalent to the enzyme $K_{m}$ for the substrate (49, 50). As discussed by Nare et al. (20), substrate inhibition of an enzyme is probably non-physiological and insignificant in *vivo* (49), primarily due to the high energetic cost required for maintaining high substrate concentrations (50). Clearly, the intracellular activity of PTR1 must be restricted to the physiological range of intracellular substrate (i.e. up to 440 nM). Based on the kinetic parameters determined for the pure recombinant enzyme at physiological pH and ionic strength, it is possible to predict the behavior of the system when H$_2$B is converted to either qH$_2$B or H$_2$B (it is not possible to determine the kinetic behavior for bipterin by either the current HPLC or spectrophotometric methods). The predicted rate behavior in the physiological substrate range for these dihydropterins is shown in Fig. 7. What is striking from this analysis is that, with qH$_2$B as substrate, where $[S] \ll K_{m}$ ($K_{m}^{app} = 3,400$ nM, Table 1) the rate is essentially first order over the entire range of possible substrate concentrations *in vivo*, whereas the enzymatic rate with H$_2$B as substrate plateaus at $\sim 100$ nM ($K_{m}^{app} = 29$ nM) due to the effects of inhibition by substrate ($K_{i} = 1.2$ µM). Enzymatic rates for both substrates are the same at $[S] = 100$ nM, where $\sim 25\%$ of the total bipterin is oxidized to either qH$_2$B or H$_2$B. Finally, it is evident from Fig. 7 that H$_2$B is the preferential substrate at low $[S]$, whereas qH$_2$B is the preferential substrate at high $[S]$. Thus, despite the markedly different kinetic parameters for PTR1 with these substrates *in vitro*, both can be predicted to behave as physiologically relevant substrates in the whole cell.

The presence or absence of a *bona fide* NADH-dependent QDPR in these trypanosomatids has important implications for drug development against these diseases (Fig. 8). Both organisms possess PTR1 and PCD, whereas *T. brucei* lacks QDPR and PAH. The presence of PCD would suggest that 4a-carbinolamine tetrahydrobipterin is being formed in these organisms. Certainly this would appear to be the case for *L. major* because conversion of phenylalanine to tyrosine by PAH would involve conversion of H$_2$B to this intermediate. However, as mentioned in the introduction, this gene is not essential for *L. major* (26). We therefore propose that additional unknown hydroxylation reactions exist in these organisms that would account for the essential requirement for pterins. This would explain why PTR1 is essential in *T. brucei* and why ptr1/− organisms can only be rescued with *LmQDPR* as an add-back in the presence of H$_2$B in the growth medium. Although PTR1 is not an attractive drug target *per se* in *L. major*, we suggest that QDPR may well be. Gene knock-out studies should be attempted to assess this prediction.

In conclusion, our studies have established that PTR1 is a potential drug target in the African trypanosome, but not in cutaneous leishmaniasis, caused by *L. major*. The kinetic parameters obtained under physiological conditions support the idea that PTR1 would operate in intact cells to regenerate H$_2$B from either H$_2$B or qH$_2$B. The origin of these metabolites is currently under study in our laboratory.

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