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Methylene tetrahydrofolate dehydrogenase/cyclohydrolase and the synthesis of 10-CHO-THF are essential in *Leishmania major*

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Summary

10-formyl tetrahydrofolate is a key metabolite in C1 carbon metabolism, arising through the action of formate-tetrahydrofolate ligase (FTL) and/or 5,10-methenyltetrahydrofolate cyclohydrolase/5,10-methylene tetrahydrofolate dehydrogenase (DHCH). *Leishmania major* possesses single *DHCH1* and *FTL* genes encoding exclusively cytosolic proteins, unlike other organisms where isoforms occur in the mitochondrion as well. Recombinant DHCH1 showed typical $NADP^+$-dependent methylene tetrahydrofolate DH and 5,10-methenyltetrahydrofolate CH activities, and the DH activity was potently inhibited by a substrate analog 5,10-CO-THF (K_i 105 nM), as was *Leishmania* growth (EC_{50} 1.1 μM). Previous studies showed null *ftl*$^-$ mutants were normal, raising the possibility that loss of the purine synthetic pathway had rendered 10-CHO-THF dispensable in evolution. We were unable to generate *dhch1*$^-$ null mutants by gene replacement, despite using a wide spectrum of nutritional supplements expected to bypass DHCH function. We applied an improved method for testing essential genes in *Leishmania*, based upon segregational loss of episomal complementing genes rather than transfection; analysis of ~1400 events without successful loss of *DHCH1* again established its requirement. Lastly, we employed 'genetic metabolite complementation' using ectopically expressed *FTL* as an alternative source of 10-CHO-THF; now *dhch1*$^-$ null parasites were readily obtained. These data establish a requirement for 10-CHO tetrahydrofolate metabolism in *L. major*, and provide genetic and pharmacological validation of DHCH as a target for chemotherapy, in this and potentially other protozoan parasites.

Keywords

trypanosomatid protozoa; C1-THF metabolism; 1 carbon transfer; chemotherapy; formyl methionyl-tRNA

Introduction

Leishmania are important pathogens infecting millions of people worldwide. The symptoms of these infections range from skin lesions and disfiguring necrosis in the face, to lethal pathology in the liver and spleen, depending on the *Leishmania* species involved. Although lasting immunity is possible, no vaccine is presently available, and the drugs used in chemotherapy suffer from various deficiencies, such as toxicity, high cost and emerging

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Supplemental information for "Methylene tetrahydrofolate dehydrogenase/cyclohydrolase and the synthesis of 10-CHO-THF are essential in *Leishmania major", by* Silvane M. F. Murta, Tim J. Vickers, David A. Scott, and Stephen M. Beverley.

resistance (Kedzierski *et al.*, 2006, Mishra *et al.*, 2007). Consequently, the development of alternative therapies is an urgent priority.

Folate derivatives are essential cellular cofactors in the synthesis of thymidine, purines and the amino acids glycine and methionine, in the metabolism of histidine and serine (Nzila *et al.*, 2005, Christensen & MacKenzie, 2006) and for mitochondrial protein synthesis through the formylation of initiator methionyl-tRNA^{Met} (Kozak, 1983). The metabolism of reduced folate cofactors is of particular interest in drug discovery, since dihydrofolate reductase (DHFR) can be efficiently targeted in anti-tumor and anti-microbial chemotherapy (Nzila et al., 2005, Schweitzer *et al.*, 1990, Then, 2004). While current antifolates are ineffective against *Leishmania* because DHFR inhibition is bypassed by pteridine reductase 1 (PTR1), which is relatively insensitive (Bello *et al.*, 1994), combined inhibitor strategies may overcome this (Hardy *et al.*, 1997, Cavazzuti *et al.*, 2008). In this work we explore a potential new arena for chemotherapy, the enzymes responsible for the synthesis of 10-formyl tetrahydrofolate (10-CHO-THF).

Three known enzyme activities mediate production of 10-CHO-THF (Fig. 1) (Christensen & Mackenzie, 2008,Christensen & MacKenzie, 2006,Appling, 1991). In one pathway formate is added directly onto THF in an ATP-dependent reaction by formate-tetrahydrofolate ligase (FTL, EC 6.3.4.3) (Rabinowitz & Pricer, 1962). Alternatively, 10-CHO-THF arises in two steps from 5,10-methylene-tetrahydrofolate (5,10-CH_2-THF), beginning with oxidation to 5,10-methenyl-tetrahydrofolate (5,10-CH=THF) by a $NADP^+$ or NAD^+-dependent methylenetetrahydrofolate dehydrogenase (DH, EC 1.5.1.5 or 1.5.1.15, respectively) (Moore *et al.*, 1974,Hatefi *et al.*, 1957). 5,10-CH=THF is then hydrolyzed to 10-CHO-THF by methenyltetrahydrofolate cyclohydrolase (CH, EC 3.5.4.9). These enzymes occur physically in various combinations, with monofunctional enzymes such as a human, bacterial and plant FTLs, bifunctional enzymes with dehydrogenase and cyclohydrolase activities (referred to as DHCH enzymes), or trifunctional enzymes with all three activities (referred to as the C1-THF synthases) (Christensen & Mackenzie, 2008,Christensen & MacKenzie, 2006,Appling, 1991,Nour & Rabinowitz, 1991,Tan *et al.*, 1977). Adding to this complexity, multiple genes often encode these activities in a single organism, with humans expressing both a trifunctional C1-synthase in the cytoplasm (*MTHFD1*), and a bifunctional DHCH (*MTHFD2*) and a monofunctional FTL (*MTHFD1L*) targeted to the mitochondrion (Christensen *et al.*, 2005b). Other eukaryotes express differing combinations of both cytosolic and organellar DHCH and FTLs (Christensen & MacKenzie, 2006,Hanson & Roje, 2001).

The two products of these enzymes have several known functions. 5,10-CH=THF is best-known as an intermediate leading to 10-CHO-THF (Hanson & Roje, 2001, Christensen & MacKenzie, 2006, Appling, 1991), although it also occurs in some DNA photolyases, and functions in mammalian histidine catabolism (Weber, 2005, Stanger, 2002). These pathways have not been described in *Leishmania* and their properties in other organisms suggest they are unlikely to be essential. 10-CHO-THF acts as a formyl donor in purine biosynthesis, and in bacteria and eukaryotic organelles for formylation of the initiator methionyl-tRNA^{Met} to produce fMet-tRNA^{Met} (Appling, 1991, Christensen & MacKenzie, 2006, RajBhandary, 1994, Varshney *et al.*, 1993, Kozak, 1983). Interestingly, many protozoans including trypanosomes and *Leishmania* lack the *de novo* purine pathway and rely exclusively on salvage (Carter *et al.*, 2008), eliminating the need for 10-CHO-THF in this capacity. The importance of initiator Met-tRNA^{Met} formylation is the subject of some controversy. While thought originally to be essential in bacteria, recent data suggest that is not always the case (Newton *et al.*, 1999, RajBhandary, 2000). Mitochondrial initiator Met-tRNA^{Met} formylation is dispensable in the yeast *Saccharomyces cerevisae* but may be required in humans; even when not essential, the consequences of its loss vary widely (Li *et al.*, 2000, Spencer & Spremulli, 2004, Vial *et al.*, 2003). Studies in the protozoal parasite *Trypanosoma brucei*, a member of

the protozoal subfamily Trypanosomatidae to which *Leishmania* belongs, show that Met-tRNA is imported into the mitochondrion and then formylated, where it is used for mitochondrial protein synthesis (Tan *et al.*, 2002, Charriere *et al.*, 2005). RNAi knockdown of the formyl-Met-tRNA^{Met} transferase (*FMT*) shows little phenotype. However, negative results in RNAi knockdowns sometimes can arise through incomplete inhibition, a particular problem in cofactor metabolism where even low levels of THF-dependent metabolites can fulfill normal metabolic needs. Importantly, simultaneous RNAi knockdown of the *T. brucei FMT* with the mitochondrial initiation factor *IF2* yield significant growth inhibition (Charriere et al., 2005), possibly signifying the importance of fMet-tRNA^{Met} for essential mitochondrial protein synthesis.

Previously we and others used database mining to survey the 10-CHO-THF synthetic pathway in *Leishmania* and other trypanosomatids (Vickers *et al.*, 2008, Opperdoes & Coombs, 2007). The *L. major* genome encodes an active monofunctional FTL and a predicted bifunctional DHCH encoded by the *DHCH1* gene. Both proteins were localized exclusively in the cytoplasm consistent with the absence of a predicted N terminal mitochondrial targeting sequence, as shown by immunoblotting of cellular fractions as well as GFP fusions to *DHCH1* (Vickers et al., 2008). Unexpectedly, *ftl*$^{-}$ null mutants showed normal viability and virulence. As noted above, the absence of purine synthesis mitigates the need for 10-CHO-THF in *Leishmania*, and potentially this could reduce or even eliminate this requirement globally.

Here we characterize the role of *DHCH1* in *L. major* metabolism through enzymatic, genetic knockout, and pharmacological tests. First we show that *DHCH1* encodes a functional protein with NADP-dependent DH and CH activities, with kinetic properties similar to those seen in other organisms. We used both traditional transfection-based and a new plasmid segregational approach to establish that *DHCH1* was essential. Notably, over-expression of FTL enabled the recovery of *dhch1*$^{-}$ mutants, establishing through 'genetic metabolite complementation' that 10-CHO-THF is essential. Lastly we showed that *Leishmania* growth and DHCH1 activity can be inhibited by a specific DHCH inhibitor. In total these data provide genetic and pharmacological validation of DHCH as a target for anti-parasitic chemotherapy.

Results

Enzymatic activity of recombinant L. major DHCH1

Previously we used database mining of trypanosomatid genomes to identify gene LmjF26.0320 as *DHCH1* as the only functional DHCH in *Leishmania major* (Vickers et al., 2008). A hexahistidine-tagged DHCH1 was expressed in *E. coli* and the recombinant protein was purified by metal-affinity chromatography (Supplementary Figure S1) (Vickers et al., 2008). Dehydrogenase activity was measured by following the conversion of 5,10-CH_2-THF to 5,10-CH=THF, while the cyclohydrolase activity was monitored by following the consumption of 5,10-CH=THF, yielding 10-CHO-THF (see methods). Purified DHCH1 showed both 5,10-CH_2-THF dehydrogenase and 5,10-CH=THF cyclohydrolase activities, with specific activities of 22 ± 2 and 6.3 ± 0.8 μmol min^{-1} mg^{-1}, respectively. In comparison to the activities of the porcine cytosolic multifunctional C1-synthase the *L. major* enzyme has a slightly higher dehydrogenase activity and a lower cyclohydrolase activity, with the porcine enzyme having activities of 7.5 and 22 μmol min^{-1} mg^{-1}, respectively (Tan et al., 1977).

The kinetic properties of the dehydrogenase reaction were determined and are summarized in Table 1. The reaction followed typical Michaelis-Menten kinetics and could be saturated with either substrate. In mammals, the cytoplasmic DH activity of the trifunctional C1-synthase is dependent on NADP+ (Tan et al., 1977), whereas the mitochondrial DH is dependent on NAD + (Mejia & MacKenzie, 1988). The *L. major* DHCH1 dehydrogenase activity was highly

dependent upon $NADP^+$, with no activity detected with NAD^+ (limit of detection 0.3 μmol min^{-1} mg^{-1}, about 1% of the activity seen with $NADP^+$). While the DH activity of human mitochondrial DHCH with NAD^+ requires phosphate and magnesium ions (Christensen *et al.*, 2005a), when tested in the presence of both 5 mM $(K^+)PO_4$ and 5 mM $MgCl_2$ *L. major* DHCH1 still showed no NAD^+-dependent activity (data not shown). These data are consistent with the conservation of residues in *Lm*DHCH1 that interact with the $NADP^+$ cofactor in the human cytosolic enzyme, notably Arg173 that interacts with the coenzyme 2' phosphate group in the human enzyme's active site (Allaire *et al.*, 1998) (Fig. S2). In comparison to the human enzymes, the *Leishmania* DHCH1 dehydrogenase activity appears most similar to that of the $NADP^+$-dependent cytoplasmic C1 synthase, with a lower K_m for the coenzyme and lower turnover than the human mitochondrial DHCH *MTHFD2* (Pawelek & MacKenzie, 1998).

Attempts to generate DHCH1 knockout by 'classic' double replacements

Leishmania are predominantly diploid and thus inactivation of most genes requires two rounds of gene replacement (Cruz *et al.*, 1991). While some chromosomes show aneuploidy in some WT *Leishmania* strains, preliminary CGH data suggest that Chromosome 26 bearing *DHCH1* is disomic in the *L. major* line studied here (E. Kruvand and S. Beverley, in preparation). Thus, we constructed two targeting fragments, with the *DHCH1* ORF replaced by ones encoding puromycin (*PAC*) or hygromycin B (*HYG*) resistance, flanked by ~ 1 kb of 5′ and 3′ sequence (Fig. 2A). Electroporation of either fragment separately into WT *L. major* followed by plating on selective media yielded transfectant colonies efficiently at control frequencies (not shown). Analysis of 20 clonal transfectant lines showed that all were heterozygotes (*DHCH1/Δdhch1::PAC* or *DHCH1/Δdhch1::HYG*, abbreviated hereafter as *PAC*/+ or *HYG*/+) as expected (Fig 2A, Supplementary Figure S3, and data not shown). These studies used PCR tests with primer pairs containing one located outside of the targeting fragment to either the 5′ or 3′ side, partnered with a marker-specific primer (Fig. S3, 2B, 2C and data not shown).

Next the *HYG*/+ or *PAC*/+ heterozygotes were submitted to a second round of electroporation with the remaining targeting fragment (*PAC* or *HYG* respectively) and plated on semisolid media containing both puromycin and hygromycin B. PCR tests of 12 doubly resistant clonal transfectant lines showed that each contained both the planned *HYG* and *PAC DHCH1* replacements (Fig 2B, 2C). Nonetheless, the clonal transfectants retained a copy of *DHCH1*, as revealed by PCR amplification of an intact *DHCH1* ORF (Fig. 2D), and grew normally (data not shown). This finding of successful 'double replacement' accompanied by retention of the WT gene has been seen previously in attempts to target other essential genes in *Leishmania*, arising through generation of aneuploid or tetraploid parasites with additional chromosomes bearing the target gene (Balana-Fouce *et al.*, 2008, Cruz *et al.*, 1993, Dumas *et al.*, 1997, Ilgoutz *et al.*, 1999a, Vergnes *et al.*, 2005). Analysis of the DNA content of the doubly-targeted lines above by flow cytometry showed WT DNA contents (data not shown), suggested that these were likely to be aneuploids, bearing at least one 'WT' chromosome along with the planned *HYG* and *PAC* replacements (Fig. 2A).

Attempts to generate DHCH1 knockout by double replacement using different nutritional supplements

We attempted to bypass the consequences of *DHCH1* ablation through metabolic complementation. In these studies, the 2nd round transfections above were repeated, but with parasites grown and plated on media containing various supplements potentially able to bypass the *DHCH1* requirement. First we included metabolites known to participate and/or arise in C1 folate metabolism, including glycine, serine, formate, tetrahydrofolate, thymidine and folinic acid. Second, parasites were grown with the supplements above plus the C1 folates 5,10-CH=THF and 10-formylfolic acid (10-CHOFA), the first being an intermediate

metabolite formed through the DH activity of DHCH1 and the second constituting a potential precursor to 10-formyl-THF. While the ideal supplement would have been 10-CHO-THF, this metabolite is very unstable; however, under the mildly alkaline pH of *Leishmania* culture media (7.4), spontaneous isomerization of 5,10-CH=THF to 10-CHO THF occurs over time (Rabinowitz, 1963, Brouwer *et al.*, 2007). Lastly, since the expected role of 10-CHO-THF in *Leishmania* arises through its role in mitochondrial protein synthesis, we attempted to bypass this potential requirement by performing transfections in the presence of pyruvate and uridine, two metabolites that have been proposed to bypass the need for mitochondrial DNA and presumably the proteins encoded therein (Sen *et al.*, 2007).

In total, 65 independent clonal lines were analyzed, all of which yielded the presumptive aneuploidy phenotype by the PCR assay described above (data not shown), i.e. parasites bearing both the planned *HYG* and *PAC* replacements while retaining at least one copy of the *DHCH1* ORF, without the recovery of any *dhch1*$^{-}$ null mutants.

Deletion of chromosomal DHCH1 in the presence of ectopically expressed DHCH1

An important control was whether successful targeting could be achieved in the presence of ectopic *DHCH1* expression, as this would render unlikely the possibility that molecular events neither anticipated nor tested in our strategy above had occurred. First, *DHCH1* was expressed from the multicopy episomal pXNG4 vector, which bears a nourseothricin (SAT) resistance marker (D. Scott and S. Beverley, in preparation). DHCH assays of WT protein extracts showed a specific activity of 1 nmol^{-1} min^{-1} mg, a value not significantly different than the assay background in controls lacking NADP+, whereas WT/pXNG4-*DHCH1* transfectants showed a specific activity of 31 nmol^{-1} min^{-1} mg, at least 30-fold greater. pXNG4-*DHCH1* was then introduced into the *HYG*/+ or *PAC*/+ heterozygotes described previously, and the second chromosomal allele in these lines targeted by transfection as before (Fig. 3A). To visualize the chromosomal *DHCH1* allele, PCR using a forward primer located 5′ and outside of the *DHCH1* targeting fragment and a reverse primer located within the *DHCH1* ORF was used (Fig. 3B).

In contrast to the results above with parasites lacking ectopic *DHCH1* expression, the chromosomal *DHCH1* was now readily lost in all 12 independent clonal transfectants tested (Fig. 3B). PCR tests showed that these lines contained both the *HYG* and *PAC* Δ*dhch1*$^{-}$ replacements, while retaining the *DHCH1* ORF borne on pXNG4-*DHCH1* (data not shown). Thus, in the presence of ectopically expressed *DHCH1*, both chromosomal *DHCH1* alleles could be successfully eliminated, yielding parasites that were genetically *dhch1*$^{-}$/+pXNG4-*DHCH1*. These methodological controls further support the conclusion that under the conditions tested, *DHCH1* is essential.

Genetic metabolite complementation permits the recovery of DHCH1 replacement

A requirement for *DHCH1* was somewhat surprising, since *Leishmania* possess a second cytoplasmic pathway for 10-CHO-THF synthesis through formyl tetrahydrofolate ligase (FTL), whose enzymatic activity has been verified (Vickers et al., 2008) (Fig. 1). This could indicate that *DHCH1* has an unanticipated function, or alternatively, that the activity of the FTL pathway was insufficient for metabolic needs in the absence of *DHCH1*. Indeed, evidence that the FTL pathway is of secondary importance comes from the fact that *ftl*$^{-}$ mutants could be readily generated without any apparent effect on growth *in vitro* or virulence in animal infections (Vickers et al., 2008).

Thus we asked whether provision of 10-CHO-THF by over-expression of FTL using the multicopy episomal vector pXNG4-*FTL* could bypass the requirement for *DHCH1* (Fig. 4A). pXNG4-*FTL* was introduced into the heterozygous +/*PAC* or +/*HYG* lines, and FTL over-

expression was confirmed by western blot analysis (Supplementary Figure S4). These parasites were then submitted to second round of targeting as before. Analysis of 12 independent transfectant lines showed that all now lacked the *DHCH1* ORF entirely, and western blot analysis with a polyclonal anti-DHCH1 antiserum confirmed a complete loss of DHCH1 protein (Fig. 4B, C). The ability of elevated *FTL* expression to enable parasites to readily dispense with *DHCH1* is strong evidence of genetic metabolite complementation, presumably through the product of FTL activity, 10-CHO-THF, and in turn argues strongly that the essential role of DHCH1 is the provision of this metabolite.

Forcing loss of pXNG4-DHCH1 by negative selection

As a final test probing the essentiality of *DHCH1* in WT *Leishmania*, we applied a method building on 'plasmid shuffling' approaches common in yeast genetics. Plasmid segregation based approaches have the advantage of allowing the separation of tests of gene function from transfection, which is relatively inefficient (D. Scott and S.M. Beverley, in preparation). In our studies this utilized two features of the episomal pXNG4 vector, namely the presence of both a negative selective marker (a modified HSV thymidine kinase gene) and a positive GFP fluorescence marker (Fig. 5A). The starting point was the *dhch1*$^{-}$/pXNG4-*DHCH1* parasite described earlier, where the chromosomal *DHCH1* was ablated and parasite growth maintained by the episomal pXNG4-*DHCH1* (Fig. 3, 5A).

dhch1^{-}/pXNG4-*DHCH1* parasites were grown briefly (24 h, or ~3 doublings) in the absence of nourseothricin (selective for the *SAT* marker of episomal pXNG4) but in the presence of ganciclovir, which through the action of HSV TK selects against parasites bearing pXNG4. Over this period little effect on parasite growth was seen. Then, parasites were subjected to flow cytometry, to assess plasmid copy number by following expression of the GFP marker. Two populations of cells were evident following this treatment: first, 'bright' cells showing strong fluorescence (>200 FU; M2 gate in Fig. 5B) and presumably high copy numbers of pXNG4-*DHCH1*; and 'dim' cells, showing background fluorescence (2–20 FU; M1 gate in Fig. 5B), presumably either lacking pXNG4-*DHCH1*, or bearing just a few copies. Controls showed that in the absence of ganciclovir the *dhch*1^{-}/+pXNG4-*DHCH1* parasites retained high GFP fluorescence, while WT cells showed background fluorescence only (Fig. 5C, 5D). As our previous results indicated that *DHCH1* was essential, we expected that the 'bright' cells retaining pXNG4-*DHCH1* would be viable, while the 'dim' cells would suffer a growth disadvantage and perhaps perish.

We used flow cytometry to select single cells into individual wells of a 96 well microtiter plate, focusing on the 'dim' (M1) or 'bright' (M2) parasites from the M1 population shown in Fig. 5B. We inoculated parasites into plates containing M199 media containing puromycin and hygromycin B (to guarantee retention of *dhch1*$^{-}$ cells) but lacking ganciclovir or nourseothricin (pXNG4 markers). Several experiments were performed with media supplemented additionally with the various metabolites described earlier.

In these studies, 478/672 of the wells inoculated with a single 'bright' parasite grew out (71 %), a value similar to that seen with controls probing the effects of sorting, recovery and growth of single cells in the cells (50 – 80%). In contrast, in wells receiving a single 'dim' parasite, growth was seen in only 48/2016 (2.4 %) (Fig. 5B). Importantly, all 48 'dim' parasite lines retained the pXNG4-*DHCH1* plasmid, as judged by their ability to grow in the presence of nourseothricin (SAT^{R}) and/or *DHCH1* PCR tests. We presume these cells arose either by imperfect sorting, and/or from cells originally expressing low levels of GFP due to the presence of only one or a few copies of pXNG4-*DHCH1*. After correcting for the intrinsic plating efficiency, we estimate that in total approximately 1430 cells (71% of 2016) had been scored for their ability to grow in the absence of *DHCH1*. Thus plasmid shuffling enabled us to extend

the stringency of our selection from a total of 65 events scored by the 'classic' transfection based approach, to a value more than 20-fold higher.

Interaction of L. major DHCH1 with novel antifolates

To corroborate the conclusion that DHCH activity was essential for *Leishmania* growth, we turned to pharmacological tests. The substrate analogue 5,10-CO-THF ((2S)-2-[[4-[(6aR)-3-amino-1,9-dioxo-5,6,6a,7-tetrahydro-4H- imidazol[3,4-f]pteridin-8-yl]benzoyl]amino] pentanedioic acid) was previously shown to be a potent inhibitor of the DHCH activity of the human cytosolic C1-synthase (Tonkinson *et al*., 1998, Temple *et al*., 1982). We predicted that this compound should also be a potent inhibitor of the *Leishmania* DHCH1, based on the overall sequence conservation, including the residues shown to intact with this inhibitor in structural studies of the human C1-synthase DHCH domain (Schmidt *et al*., 2000) (Fig. S2). Indeed, 5,10-CO-THF was a potent inhibitor of the DH activity of recombinant *Leishmania* DHCH1, with the data fitting to a simple competitive model with respect to 5,10-CH_2-THF, giving a K_i of 105 ± 2 nM (Fig. 6). This was about 10-fold higher than the K_i of 18 ± 5 nM seen for the DH activity of the human cytosolic C1-synthase (Schmidt et al., 2000). While 5,10-CO-THF was shown previously to be a weak inhibitor of the human DHFR (Ki > 100 μM) (Temple et al., 1982), 100 μM 5,10-CO-THF did not inhibit the activity of *Leishmania* DHFR-TS (data not shown) nor FTL (Vickers et al., 2008). *L. major* DHCH1 was also not inhibited by pteridine analogues shown previously to inhibit *L. major* DHFR or PTR1, including methotrexate or hydrophobic diamino-quinazoline, -pyrimidine or -pteridine analogues (compound 34: 2,4-diamino-6-(3,4-dichlorophenoxy)-quinazoline); compound 70; 2,4-diamino-6-benzyl-5-(3-phenylpropyl)-pyrimidine and compound 25 (2,4-diamino-6,7-diisopropylpteridine) (Hardy *et al*., 1997, Bello et al., 1994) (tested at 50 μM; data not shown).

Toxicity of folate analogues to cell lines

5,10-CO-THF inhibited *L. major* growth with an EC_{50} of 1.1 ± 0.04 μM (Table 2). Parasites overexpressing *DHCH1* (WT/pXNG4-*DHCH1* or *dhch1*$^-$/+pXNG4-*DHCH1*) showed 4–5 fold resistance ($p < 0.0001$ and $p < 0.0003$, respectively; Table 2), consistent with the studies of purified DHCH above. Parasites overexpressing *FTL* (WT/pXNG4-*FTL* or *dhch1*$^-$/pXNG4-*FTL*) also showed 3-fold resistance ($p < 0.0001$ and $p < 0.0002$, respectively; Table 2), implying that FTL activity could act as a metabolic by-pass for DHCH inhibition. Consistent with the enzyme inhibition data, *DHCH1* or *FTL* overexpressors did not show resistance to methotrexate or two of the hydrophobic antifolates tested above (compounds 34 and 70; Table 2). Interestingly, even in the absence of a DHCH target (*dhch1*$^-$/pXNG4-*FTL* line), growth was inhibited with an EC_{50} in the μM range (Table 2), suggesting 5,10-CO-THF has other targets in *Leishmania.* FTL can be excluded as it is not sensitive to 5,10-CO-THF, and while the bridged structure of the inhibitor does not closely resemble that of 10-CHO-THF, it seems probable that the target could be other folate-dependent enzymes or transporters within the cell.

Discussion

We show here that *L. major* expresses an active bifunctional $NADP^+$-dependent DHCH encoded by *DHCH1* (Table 1). Previous studies have localized DHCH1 protein to the parasite cytosol, consistent with the predicted lack of an N terminal mitochondrial targeting sequence and the NADP dependency of the DH activity (Vickers et al., 2008). Attempts to generate *dhch1*$^-$ null mutants by homologous replacement were unsuccessful, despite analysis of a large number of independent events and the use of metabolic supplements shown in other organisms to rescue DHCH-deficiency. Instead, these studies invariably yielded parasites bearing the two planned replacements, but also bearing extra copies of *DHCH1* that most likely arose through aneuploidy. The unusual *Leishmania* phenotype of the recovery of planned replacements, but

with retention of the target gene through aneuploidy or tetraploidy, is widely used as an indication that a gene is gene essential in this organism. As a technical control, chromosomal *dhch1*$^{-}$ replacements were readily obtained after ectopic expression of *DHCH1*. Lastly, we applied a plasmid shuffling approach that allowed us to increase the number of events analyzed 20 fold: from 65 by classic transfectional approaches, to more than 1,400 through negative selection and cell sorting. However, we were still unable to recover a viable *dhch1*$^{-}$ null mutant (the virtues and potential of the 'plasmid shuffle' methodology in *Leishmania* are discussed further below). Thus we are confident in concluding that *DHCH1* is an essential gene in *L. major* under these circumstances.

Notably, we were able to completely remove *DHCH1* following ectopic expression of FTL, which provides an alternative metabolic route to 10-CHO-THF synthesis via the formate tetrahydrofolate ligase activity. This form of 'genetic metabolic complementation' suggests that parasite dependency on *DHCH1* activity arises through the provision of its ultimate metabolite of both FTL and DHCH1, 10-CHO-THF. Hypothetically, the active metabolite could be 5,10-CH=THF, as these two metabolites interconvert at significant rates under physiological conditions, which also precludes measurement of their individual abundance in the cell (Brouwer et al., 2007). However, as yet there is no known essential role in metabolism for 5,10-CH=THF, other than as an intermediate in 10-CHO-THF synthesis, and we thus favor the conclusion that 10-CHO-THF is the relevant metabolite.

Interestingly, the levels of WT *FTL* activity appear insufficient to rescue the *dhch1*$^{-}$ parasites, consistent with our previous finding that null mutant *ftl*$^{-}$ parasites were phenotypically normal when grown *in vitro* and are fully infective to susceptible mice (Vickers et al., 2008). Hypothetically, it is possible that elevated *FTL* synthesis or other alterations in 10-CHO-THF metabolism could bypass the requirement for *DHCH1* under some circumstances, and indeed in other studies we have been able to detect second site mutations including *FTL* gene amplification in other selections forcing loss of *DHCH1* (S. Murta and S. Beverley, unpublished data).

What is the role of 10-CHO-THF in Trypanosomatid metabolism?

In mammals, known roles of 10-CHO-THF include the provision of formyl groups in purine biosynthesis, as an intermediate C1-THF interconversions involved in amino acid metabolism (glycine, methionine, histidine, and serine), or the production of fMet- tRNA^{Met} in the mitochondrion (Appling, 1991, Christensen & MacKenzie, 2006). However, *Leishmania* are well known to be purine auxotrophs as their genome lacks the purine biosynthetic enzymes glycinamide ribonucleotide (GAR) and phosphoribosylaminoimidazolecarboxamide (AICAR) transformylases (EC 2.1.1.2 and 2.1.2.3 respectively) (Carter et al., 2008). Similarly, several studies have established that *Leishmania* still require the amino acids arising through the C1-pathway for normal growth (Scott *et al.*, 2008, Vickers et al., 2008). Thus under the conditions tested, formylation of the initiator Met-tRNA^{Met} currently stands as the only candidate essential metabolic role. As discussed earlier, the extent to which fMet- tRNA^{Met} synthesis is required is a matter of some debate, with differences seen in studies both within and between species in both eukaryotes and prokaryotes. However, the conclusion that *Leishmania* require fMet-tRNA^{Met} would be consistent with the finding that the related trypanosomatid parasite *T. brucei* requires formylated initiator methionyl-tRNA^{Met} for mitochondrial protein synthesis for survival (Tan et al., 2002, Charriere et al., 2005). This would then suggest that cytoplasmic 10-CHO-THF arising from either FTL or DHCH1 activity in the cytosol must then be transported to the mitochondrion. While generally it is thought that metabolites other than reduced folates are the principal route of communication for cytosolic-mitochondrial C1-dependent pathways in mammalian cells (Appling, 1991), some studies

point to trafficking of C1 tetrahydrofolates between the cytosol and mitochondrion in plants (Prabhu *et al.*, 1998, Hanson *et al.*, 2000).

The interpretations above rest upon the strength and accuracy of current genome-based models of Trypanosomatid metabolomes. Given the substantial number of hypothetical proteins of unknown function encoded in Trypanosomatid genomes (Peacock *et al.*, 2007, Ivens *et al.*, 2005, El-Sayed *et al.*, 2005, Berriman *et al.*, 2005), one cannot exclude the possibility that *Leishmania* possess novel essential pathways that require 10-CHO-THF. Indeed, the *L. major* genome predicts a highly degenerate pseudogene (pse*DHCH2*) and DHCH2-related sequences are completely absent in the *L. braziliensis* and African trypanosome genomes (Peacock et al., 2007, Ivens et al., 2005, El-Sayed et al., 2005, Berriman et al., 2005). In contrast, seemingly intact *DHCH2*s occur in *L. infantum*, *L. mexicana*, *Crithidia fasciculata* and *Trypanosoma cruzi*, whose predicted proteins show conservation of DHCH active site residues (Vickers et al., 2008). Interestingly, a GFP tagged *Li*DHCH2 is localized to the mitochondrion when expressed in *L. donovani* (Vickers et al., 2008), although we have not been able to demonstrate DHCH activity associated with over-expression of WT *LiDHCH2* (data not shown). Potentially the occurrence of a catalytically active *DHCH2* could signify an increased importance of mitochondrial 10-CHO-THF synthesis in some trypanosomatid species. In the future, direct studies of mitochondrial fMet-tRNA^{Met} synthesis mediated by FMT and whether DHCH2 is enzymatically active in those trypanosomatids that possess it will test these hypotheses.

Plasmid segregation and shuffling as an improved test of gene function

Currently, the standard approach to determining if a gene is essential in *Leishmania* is an inability to delete both chromosomal alleles by homologous gene replacement (Cruz et al., 1991, Barrett *et al.*, 1999). Fortunately, these studies are facilitated by the fact that homologous recombination occurs far more frequently than non-homologous insertions in trypanosomatids (Beverley, 2003, Clayton, 1999). Ignoring technical failures, the general experience of most investigators has been that attempts to delete essential genes in *Leishmania* yield parasites bearing the planned allelic replacements but containing additional gene copies that arise via aneuploidy or polyploidy (Cruz et al., 1993, Dumas et al., 1997, Ilgoutz et al., 1999b, Vergnes et al., 2005, Balana-Fouce et al., 2008). This is often considered a diagnostic criterion, especially when accompanied by successful replacement in the presence of an ectopic gene.

One challenge of the gene replacement-based approach is the relative inefficiency of transfection (10^{-4} or less), limiting the number of events that can be scored. For example, here we analyzed 65 events targeting the last copy of *DHCH1*, a number exceeding that typically reported in tests of *Leishmania* gene function. Other factors impinging on replacement-based approaches include that transfection efficiencies can vary greatly depending on the locus and targeting construct used (Papadopoulou & Dumas, 1997), and that survivors can also emerge through mutations unrelated to targeted gene (unpublished data). In contrast, in the plasmid shuffling approach, functional tests arise through segregation of an ectopically expressed gene borne on a plasmid bearing both positive (GFP) and negative (TK) markers, in a line previously engineered to lack the gene of interest (Fig. 5). Following a brief period of culture to allow segregation of the episomal vector, a large number of candidate ‘segregants’ can be obtained through appropriate manipulations of the GFP or TK markers, and rapidly tested. This allowed us to rapidly score ~1400 such segregants for *DHCH1*, at least 20 times the number achieved by traditional methods, and if necessary many more could have been tested. Thus we believe that the plasmid segregation method provides a significant improvement to tests of essential gene function in *Leishmania*, as first established in the yeast *Saccharomyces cerevisiae* (Forsburg, 2001). In the future one can envisage a number of other applications based on the approach of ‘plasmid shuffling’.

DHCH1 and 10-CHO-THF metabolism as a target for anti-parasitic chemotherapy

L. major folate metabolism shows many differences from that of their human hosts, including a bifunctional dihydrofolate reductase-thymidylate synthase (DHFR-TS) (Coderre *et al.*, 1983), a novel pteridine reductase (PTR1) (Bello et al., 1994), a large and complex family of folate and biopterin transporters (Ouellette *et al.*, 2002), and cytosolic but not mitochondrial synthesis of 10-CHO-THF (Vickers et al., 2008), amongst others. These factors contribute to profound differences in antifolate sensitivity; for example, the relative insensitivity of PTR1 to many antifolates and its ability to act as a metabolic bypass of DHFR inhibition accounts for the relative lack of efficacy of these drugs against *Leishmania* (Bello et al., 1994, Nare *et al.*, 1997).

While some folate metabolizing enzymes are essential in *Leishmania* (DHFR-TS, folylpolyglutamyl-synthase) (Cruz & Beverley, 1990, El Fadili *et al.*, 2002), others tested appear largely or wholly dispensable for growth and virulence, including methylene tetrahydrofolate reductase and the glycine cleavage complex (Scott et al., 2008, Vickers *et al.*, 2006). This may reflect the ability of the parasite to salvage sufficient quantities of many C1-THF dependent metabolites such as amino acids and purines (Carter et al., 2008, Scott *et al.*, 1987), particularly in the mammalian stages where the parasite reside within phagolysosomes that are postulated to be rich in free amino acids (McConville *et al.*, 2007). In contrast, we were unable to rescue the requirement for *DHCH1* with any of a wide array of metabolites known or postulated to rescue DHCH deficiency. This could reflect many factors including a deficiency in a non-salvageable metabolite, a conclusion consistent with an essential requirement for mitochondrial fMet-tRNA^{Met}.

For a parasite enzyme to be a good drug target it must be both essential for parasite survival, and dispensable, absent, or substantially different in the host (Fairlamb, 2002). In mammals both the cytosolic and mitochondrial forms of DHCH (*MTHFD1* and *MTHFD2* respectively) are essential for early development (Di Pietro *et al.*, 2002, Brody *et al.*, 2002, Parle-McDermott *et al.*, 2005). However, human fibroblasts lacking either enzyme are viable, but are purine or glycine auxotrophs (Christensen et al., 2005b, Patel *et al.*, 2003), and 10-CHO-THF metabolism is dispensable in the yeast *S. cerevisiae* when grown in rich media (West *et al.*, 1996). This suggests that the adult tissues of the human host may be less dependent upon DHCH activity than *Leishmania*. In contrast, the 10-CHO-THF pathway is simplified in many parasitic protozoans, where many species synthesize a single cytosolic DHCH and FTL (*L. major*, *L. braziliensis*), while others synthesize just a cytosolic DHCH (African trypanosomes) (Vickers et al., 2008). Preliminary genomic analysis suggest that like African trypanosomes, the malaria parasite *P. falciparum* also possess a single route to 10-CHO-THF synthesis, via a bifunctional DHCH (Nzila et al., 2005). These data suggest that the relative simplicity of the 10-CHO-THF pathway in parasitic protozoa relative to their human host may render them more sensitive to chemotherapeutic interventions.

While few DHCH inhibitors have been developed, *L. major* DHCH1 activity and promastigote growth *in vitro* were sensitive to the substrate analog 5,10-CO-THF (Temple et al., 1982). The specificity of this inhibitor was supported by the findings that *DHCH1* overexpressors are less susceptible, and it fails to inhibit DHFR or FTL enzymatic activity. However, over-expression of FTL did not completely overcome 5,10-CO-THF inhibition suggesting it could have other targets beyond *DHCH1*.

Consistent with the genetic studies above, DHCH inhibitors have little activity or toxicity against mammalian cells *in vitro*, or in mouse cancer models, at concentrations below 10 μM (Tonkinson et al., 1998, Temple et al., 1982). In preliminary studies we found modest activity of 5,10-CO-THF against *Leishmania* amastigotes in macrophages (EC_{50} ~ 5 μM), although host cell toxicity was seen above 10 μM (S. Hickerson and S. Beverley, unpublished data).

These results are encouraging since 5,10-CO-THF is actually more selective for the host than parasite enzymes (K_i = 18 v. 105 nM respectively; (Schmidt et al., 2000) Fig. 6). While highly conserved, numerous differences between *Leishmania* DHCH1 and the human cytosolic and mitochondrial DHCHs (Fig. S2) could be exploited to design more selective and potent inhibitors. DHCH inhibitors have not received a high priority in cancer chemotherapy programs due to the poor efficacy for the first ones tested, although a number of antifolates targeting 10-CHO-THF utilizing enzymes such as GAR transformylase have been developed (Zhang *et al.*, 2003). Possibly, similar pharmacophores may hold promise in targeting 10-CHO-THF metabolism in *Leishmania* and other Trypanosomatids.

EXPERIMENTAL PROCEDURES

Reagents

(6-*R*,*S*)-Tetrahydrofolate (THF) and 5,10-CH=THF were obtained from Schircks Laboratories (Jona, Switzerland). (2S)-2-[[4-[(6aR)-3-amino-1,9-dioxo-5,6,6a,7-tetrahydro-4H- imidazo [3,4-f]pteridin-8-yl]benzoyl]amino]pentanedioic acid (5,10-CO-THF) was custom synthesized by SAFC Pharma, a division of Sigma-Aldrich Corporation, according to the method of Temple *et al*. (Temple et al., 1982); the compound was 96.2% pure by high-performance liquid chromatography. Methotrexate and compound 25 (2,4-Diamino-6,7-diisopropylpteridine) were purchased from Sigma, the folate analogues compound 34 (2,4-diamino-6-(3,4-dichlorophenoxy)-quinazoline) and compound 70 (2,4-diamino-6-benzyl-5-(3-phenylpropyl)-pyrimidine) were as described (Hardy et al., 1997). Hygromycin B (HYG) was purchased from Calbiochem (EMD Biosciences, San Diego, CA) and nourseothricin (SAT) was from Werner BioAgents (Jena, Germany). All other chemicals and reagents were of analytical grade and purchased from Sigma-Aldrich.

Parasite growth and drug inhibition

All studies used derivatives of *L. major* clone Friedlin V1 promastigotes, grown at 27°C in M199 medium (US Biologicals) supplemented with 40 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.4, 50 μM adenosine, 1 μg ml^{-1} biotin, 5 μg ml^{-1} hemin, 2 μg ml^{-1} biopterin and 10 % (v/v) heat-inactivated fetal calf serum. Growth was determined by seeding parasites at 5×10^5 cells ml^{-1} at various concentrations of drug and counting cells using a model Z1 Coulter counter when cultures reached late-log phase. The EC_{50} value is the concentration of drug required to decrease growth by 50%.

Cloning of *L. major* DHCH1—The *DHCH1* coding sequence was amplified with recombinant *Pfu* DNA polymerase (Stratagene) using *L. major* genomic DNA template, prepared by the LiCl mini-prep method (Medina-Acosta & Cross, 1993), using primers SMB3528 and SMB3529, which added 5′ *Nde*I and 3′ *Bam*HI sites, respectively. The amplified product was cloned into pCR.blunt (Invitrogen), yielding pCR.blunt. *DHCH1* (B6255) and the NdeI-BamHI fragment transferred into the corresponding sites of pET15b (Novagen), yielding pET15b-*LmjDHCH1* (B6026).

Expression, purification and enzymatic assays of recombinant *L. major* DHCH1

Protein was expressed in *E. coli* from pET15b-*LmjDHCH1* and purified by metal-affinity chromatography, as described in supplemental methods. During the purification and storage of this protein the inclusion of 10% glycerol in all buffers was required to prevent loss of activity. In this manner, the enzyme could be flash-frozen with no loss in activity. DHCH activities were measured spectrophotometrically, using a temperature-controlled Beckman DU-640 spectrophotometer, essentially as described by Tan *et al.* (Tan et al., 1977) following either the formation of 5,10-CH=THF from 5,10-CH_2-THF (DH activity) or the conversion of 5,10-CH=THF to 10-CHO-THF (CH activity). 5,10-CH_2-THF dehydrogenase assays were

carried out in a 0.5 ml volume at 27°C and contained 25 mM MOPS, pH 7.3, 250 μM THF, 2.5 mM formaldehyde, 1 mM $NADP^+$ and 30 mM 2-mercaptoethanol. Assays were preincubated for 5 min to allow formation of 5,10-CH_2-THF and reactions initiated by the addition of enzyme. The reactions were then stopped after 5 min by the addition of an equal volume of 1 M HCl and the 5,10-CH=THF produced quantified at 350 nm, using a extinction coefficient of 24.9 mM^{-1} cm^{-1}. DH activity was expressed as μmoles 5,10-CH=THF produced per minute. Formaldehyde stocks were freshly-prepared from paraformaldehyde, and THF dissolved just before use in 250 mM triethanolamine-Cl, pH 7, 40 mM 2-mercaptoethanol. Cyclohydrolase activity was measured in 0.5 ml assays at 27°C containing 25 mM MOPS, pH 7.3, 30 mM 2-mercaptoethanol and 50 μM 5, 10-CH=THF. The hydrolysis of 5,10-CH=THF was followed at 355 nm, and the non-enzymatic rate subtracted.

Enzyme inhibition was measured using the dehydrogenase assay, with stocks of hydrophobic diaminoquinazolines and diaminopteridines dissolved in DMSO, and 5,10-CO-THF dissolved in 250 mM triethanolamine-Cl, pH 7, 40 mM 2-mercaptoethanol, as with THF, and stored at −80 °C. 5,10-CO-THF was custom synthesized by Sigma-Aldrich Chemical Pvt. Ltd. (Bangalore, India); the authenticity of the procuct was supported by infrared spectroscopy and proton NMR and it was estimated to be greater than 96% pure. Data from 5,10-CO-THF inhibition assays were fitted to competitive, non-competitive, uncompetitive and mixed inhibition models by non-linear regression using Sigmaplot. The DHFR activity of the bifunctional *L. major* DHFR-TS was measured as described (Hardy et al., 1997). Activity in all other drug assays was expressed as the percentage of a no-drug DMSO control.

DHCH1 replacement constructs

Fusion PCR (Szewczyk *et al.*, 2006) was used to generate the two deletion constructs, which contained either puromycin (PAC) or hygromycin (HYG) drug-resistance cassettes between the 5′ and 3′ *Lmj*DHCH1 flanking sequences (Szewczyk et al., 2006). Briefly, the flanking sequences and drug resistance cassettes were amplified separately by PCR using primers that produce overlapping ends. The 5′ and 3′ *LmjDHCH1* flanking sequences (743 and 726 bp), were amplified using the primers SMB2772/2656 and 2657/2773 that added a *Sph*I and *BamH*I site, respectively. PAC and HYG drug-resistance cassettes (600 and 1026 bp) were amplified using the primers SMB2557/2558 and SMB2561/2562, respectively that added a linker sequence. Each deletion construct was then made by mixing the purified PCR products for the flanking sequences and a drug-resistance cassette and using these products as templates in a second round of PCR. The two PCR reactions to generate the PAC and HYG constructs both used the external primers SMB2772 and 2773, and fused the flanking sequences to the correct drug resistance cassette. These two deletion constructs were then cloned into pGEM-T (Promega), yielding pGEM-T.5′. PAC.3′. *LmjDHCH1* (B4827) and pGEM-T.5′. HYG.3′. *LmjDHCH1* (B4827). The sequences of these constructs were confirmed by restriction mapping and sequencing.

DHCH1 replacement and transfections

DHCH1-targeting fragments were digested with *Sph*I and *BamH*I, and the linear targeting fragments containing PAC (2.1 kb) or HYG (2.5 kb) were transfected separately into wild-type (WT) *L. major* FV1 promastigotes as described (Robinson & Beverley, 2003). Heterozygous clones, *DHCH1/*Δ*dhch1::PAC* or *DHCH1/*Δ*dhch1::HYG* (referred as *PAC/+* or *HYG/+*), were isolated by plating on semisolid M199 medium containing 30 μg ml^{-1} puromycin or 50 μg ml^{-1} hygromycin B, respectively. Heterozygous *PAC/+* or *HYG/+* clones were transfected with deletion constructs containing *HYG* or *PAC* cassettes. The parasites were plated in semisolid M199 medium containing 30 μg ml^{-1} puromycin and 50 μg ml^{-1} hygromycin. In attempts to rescue the growth of $DHCH1^{-/-}$ homozygotes several different mixtures of different supplements were added to the plating medium. The basic supplements were: glycine

(1 mg ml^{-1}), serine (0.1 mg ml^{-1}), formate (5 mM), THF (5 μM), thymidine (10 μg ml^{-1}) and folinic acid (10 μg ml^{-1}). Additional supplements were added to the basic mixture in other plates, these were: 5,10-CH=THF (10 μM), 10-formylfolic acid 10-CHOFA (10 μM) or, alternatively, uridine (0.1 mg ml^{-1}) and sodium pyruvate (1 mM). DNA contents of permeabilized RNAse treated cells were measured by propidium iodide staining using flow cytometry (Cruz et al., 1993).

PCR primers for assessing chromosomal DHCH1 or PAC or HYG replacements

Generation of the planned *PAC* or *HYG* replacements was confirmed by PCR, using a primer located 5′ (SMB2731) or 3′ (SMB2771) of the *DHCH*1 ORF targeting fragment and a primer located within the PAC or HYG genes (SMB2557/2558 or SMB2561/2562). The loss of the *DHCH1* ORFs was confirmed using primers specific for the chromosomal *DHCH1* allele (SMB3125/3126) or using a forward primer located in the chromosome 5′ (SBM2731) of the *DHCH1* ORF targeting fragment and a reverse primer located within the *DHCH1* gene (SMB3126).

pXNG4 expression constructs

DHCH1 and *FTL* ORFs were amplified with primers (SMB3125/3126 and SMB3123/3124, respectively) from *L. major* genomic DNA, adding flanking *Bgl*II sites, and a CCACC sequence preceding the initiation codon. The *DHCH1* and *FTL* ORFs were inserted into pGEM-T (Promega) yielding pGEM-DHCH1 (B6029) and pGEM-FTL (B6030); the *DHCH1* and *FTL* ORFs were then extracted by *Bgl*II digestion and inserted into the *BglII* site of pXNG4 (B5840) to create pXNG4-DHCH1 (B6031) and pXNG4-FTL (B6032).

These plasmids were transfected into either wild-type *L. major* FV1, or heterozygote lines (*DHCH1*/Δ*dhch1::PAC* or *DHCH1*/Δ *dhch1:: HYG*) and transformants isolated as before, using plating media containing 100 μg ml^{-1} nourseothricin and either 30 μg ml^{-1} puromycin or 50 μg ml^{-1} hygromycin. The presence of plasmid was confirmed by PCR and FACS analysis for the presence of SAT and GFP markers, respectively. Three colonies over-expressing FTL or DHCH1 were submitted to second replacement using either the PAC or HYG targeting fragments. The parasites were isolated by plating on semi-solid M199 media containing 100 μg ml^{-1} nourseothricin, 30 μg ml^{-1} puromycin and 50 μg ml^{-1} hygromycin.

Single-cell sorting

Prior to flow cytometry, *dhch1*$^{-}$/pXNG4-DHCH1 lines were incubated with 50 μg ml^{-1} ganciclovir in M199 media for 24 h. Single cells sorting was then performed based upon their GFP fluorescence as described in the Results. Cells were resuspended in phosphate-buffered saline, and filtered through CellTrics 50 μm filters to remove clumps (Partec). Single cells were then recovered using a Dako MoFlo high-speed cell sorter and placed into individual wells of 96-well plates, each containing 150 μl M199 medium. Plates were incubated at 27° C for 1–2 weeks and parasite growth was scored.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

THF
tetrahydrofolate

10-CHO-THF
10-formyl tetrahydrofolate

5,10-CH=THF
5,10-methenyl-tetrahydrofolate

5,10-CH_2-THF
5,10-methylene-tetrahydrofolate

DHCH1
gene encoding *Leishmania* cytosolic methenyltetrahydrofolate cyclohydrolase/5,10-methylenetetrahydrofolate dehydrogenase

FTL
formate-tetrahydrofolate ligase

DHFR-TS
dihydrofolate reductase-thymidylate synthase

WT
wild-type

DH
dehydrogenase

CH
cyclohydrolase

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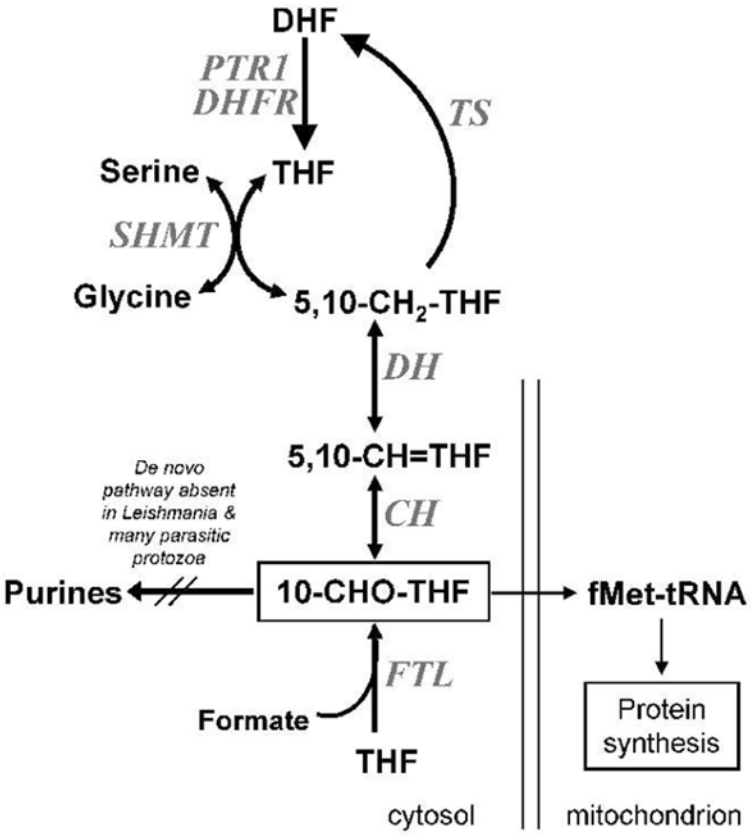

FIG. 1. 10-formyl-THF metabolism in *Leishmania*

Following synthesis through the activity of dihydrofolate reductase (DHFR, encoded by the bifunctional DHFR-TS) or pteridine reductase 1 (PTR1), tetrahydrofolate (THF) can enter C1 metabolic pathways by direct formylation in an ATP-dependent reaction by formate-tetrahydrofolate ligase (FTL, LmjF30.2600). Alternatively, THF is converted into 5,10-methylene-tetrahydrofolate (5,10-CH_2-THF) through the activity of serine hydroxymethyltransferase (SHMT). 10-CHO-THF can be generated in two steps from 5,10-methylene-tetrahydrofolate (5,10-CH_2-THF), through oxidation to 5,10-methenyl-tetrahydrofolate (5,10-CH=THF) by a $NADP^+$-dependent methylenetetrahydrofolate dehydrogenase (DH, LmjF26.0320), and hydrolysis to 10-CHO-THF by methenyltetrahydrofolate cyclohydrolase (CH, LmjF26.0320). In *L. major* the DH and CH activities are contained in the single bifunctional protein DHCH1, which like FTL is localized exclusively to the cytosol (Vickers et al., 2008). While 10-CHO-THF is used in the *de novo* purine synthetic pathway in many organisms, this pathway is absent in *Leishmania* and many parasitic protozoans (Carter et al., 2008). Current data suggest 10-CHO-THF may be utilized in the mitochondrion for methionyl-tRNA formylation, the only unique function thus far uncovered by genomic and metabolic studies in *Leishmania*.

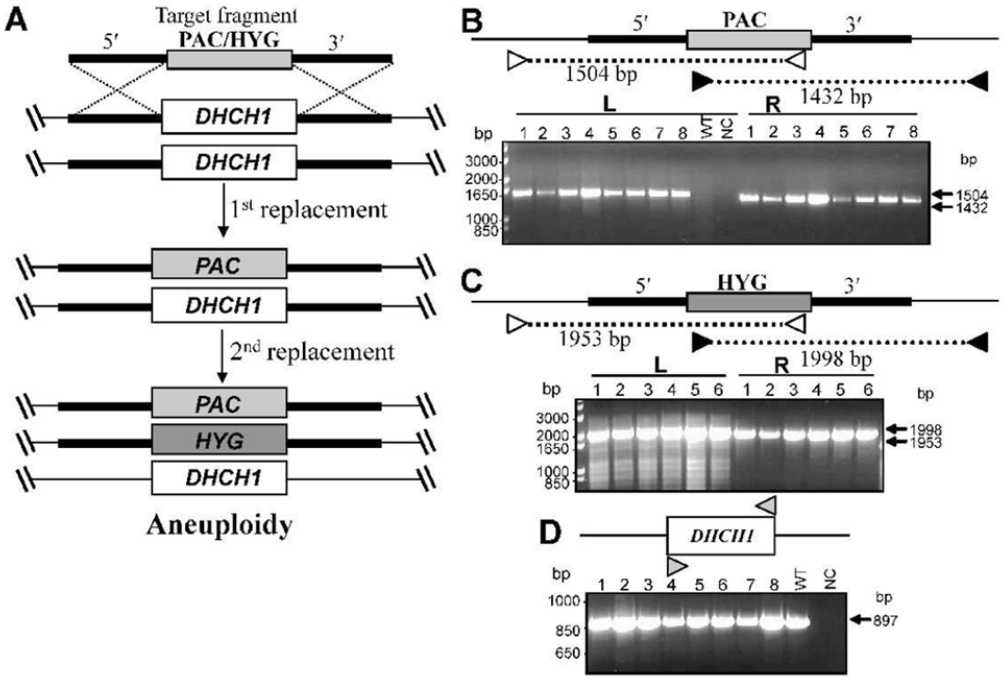

FIG. 2. Attempts to generate *dhch1*⁻ null mutants by 'classic' double replacements

A. Planned deletion of the *DHCH1* ORF by homologous gene replacement using targeting fragments with *PAC* or *HYG* resistance markers. In the first round, parasites were transfected with either construct separately; in this diagram the round starting with *PAC* is shown. Heterozygous replacement lines were obtained with high efficiency. In the second round, the +/*PAC* heterozygote (formally, Δ*dhch1::PAC/DHCH)* was transfected with the *HYG* targeting fragment, with the expectation of yielding homozogous *dhch1*⁻ null mutants. Instead, only aneuploid parasites showing both the two planned replacements but retaining WT *DHCH1* were recovered (formally, Δ*dhch1::PAC/DHCH/*Δ*dhch1::SAT/*Δ*dhch1::PAC/DHCH1)*. B. PCR tests of planned *PAC* replacements at DHCH1. Homologous replacement was visualized by PCR amplification using a primer directed against sequences located with the *PAC* ORF, and a primer located on the *DHCH1* chromosome outside the targeting fragment (indicated by heavy line) on the 5′ (white arrows) or 3′ (black arrow) side (SMB2731/2558 and SMB2771/2557, Table S1). The sizes of the predicted PCR products are shown. WT, wild-type DNA; NC, negative control (no DNA template), #1–8, eight independent lines with *PAC* replacements at *DHCH1*. C, PCR tests of planned *HYG* replacements at *DHCH1*. This experiment is similar to that described in panel B, except that primers were directed against sequences located in the *HYG* targeting fragment (SMB2731/2562 and SMB2771/2561, Table S1). #1–6, six independent lines with *HYG* replacements at *DHCH1*. D. PCR tests showing retention of DHCH1 in second round transfectants. Amplifications were performed using primers directed against sequences within the *L. major* DHCH1 ORF (SMB3125/3126, Table S1). Lines # 1 – 4 represent double replacement with P*AC/HYG* and lines # 5 – 8 with *HYG/PAC.*

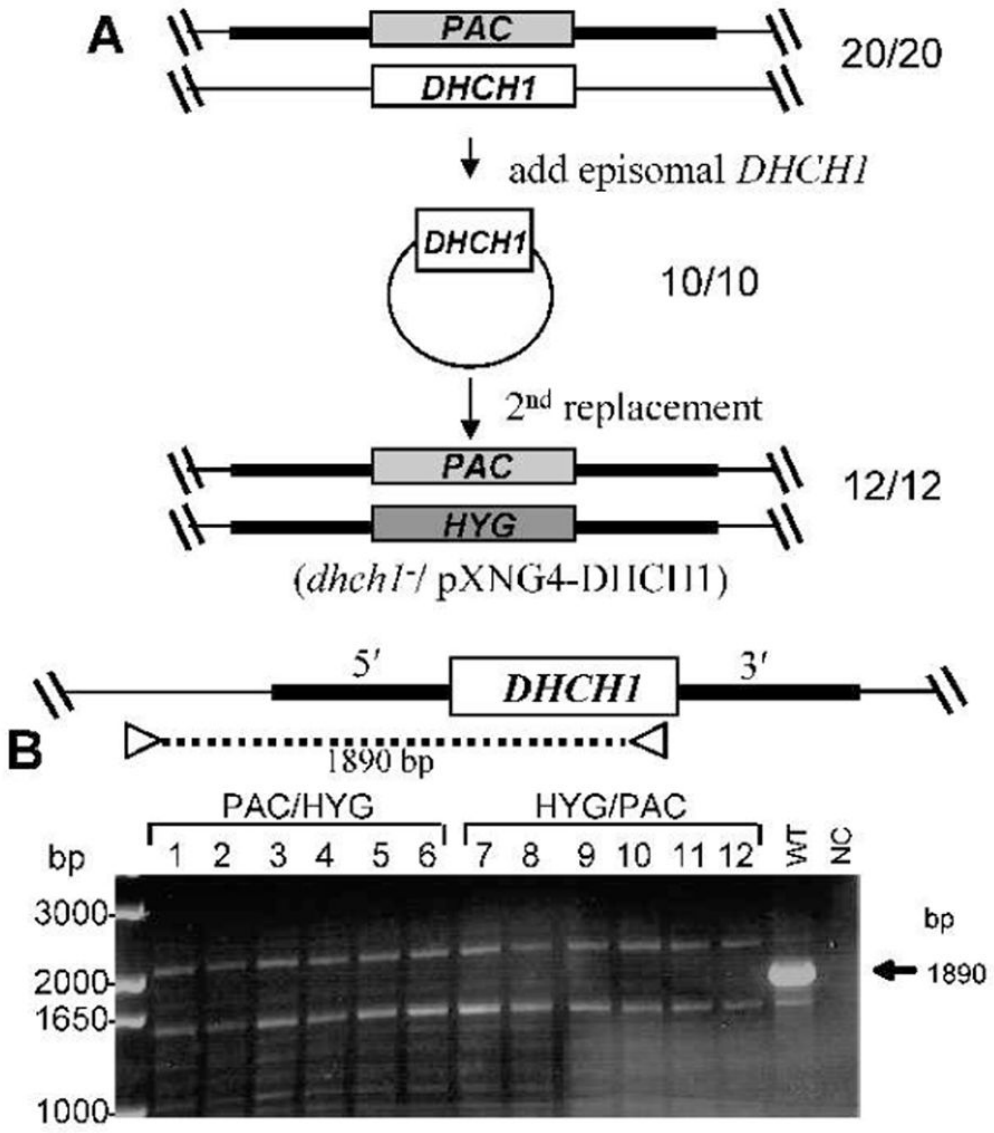

FIG. 3. Deletion of chromosomal DHCH1 in the presence of ectopic DHCH1

A. Scheme for the deletion of chromosomal *DHCH1* in the presence of ectopically expressed *DHCH1*. First, a pXNG4-*DHCH1* episomal expression construct was transfected into a *DHCH1*/Δ*dhch1::PAC* heterozygote described in Fig. 2A; 10/10 transfectants analyzed were confirmed to have the predicted genotype depicted. These *DHCH1*/Δ*dhch1::PAC* [pXNG4-DHCH1] lines were then transfected with the HYG DHCH targeting fragment and selected for resistance to all three antibiotics; of the 12 clonal lines tested, all showed the planned replacement of the chromosomal *DHCH1* locus (formally, Δ*dhch1::HYG DHCH1*/ Δ*dhch1::PAC* [pXNG4-DHCH1]).

B – PCR tests showing loss of the chromosomal *DHCH1*. To distinguish the ectopic DHCH1 from the chromosomal locus, DNA from candidate lines was subjected to PCR tests using a using a primer directed against sequences located with the DHCH1 ORFt, and a primer located on the DHCH1 chromosome outside of the targeting fragment on the 5′ side (SMB2731/3126,, Table S1). WT, wild-type DNA control; NC, negative control lacking any DNA template. The

arrow marks the size of the predicted fragment found in WT DNA, which is absent in the chromosomal *dhch1*$^{-}$ knockouts.

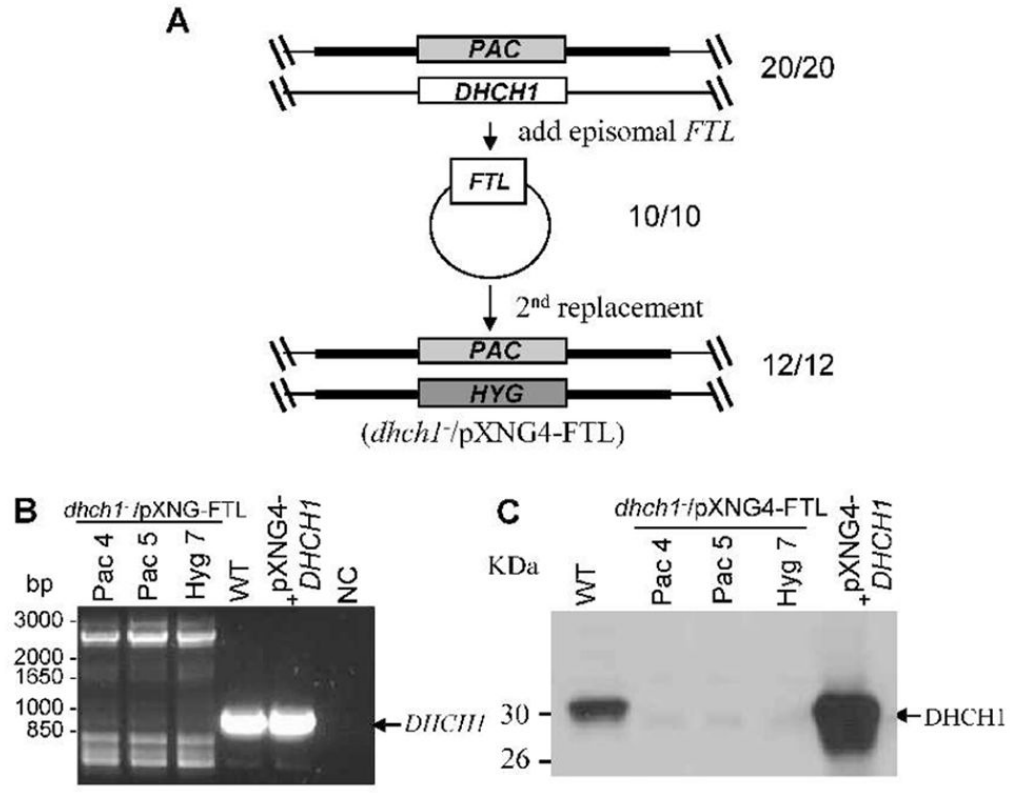

FIG. 4. Deletion of chromosomal *DHCH1* in the presence of ectopic *FTL* overexpression

A. Scheme for the deletion of chromosomal *DHCH1* in the presence of ectopically expressed *FTL*. A pXNG4-*FTL*episomal expression construct was transfected into a *DHCH1*/*PAC* (*DHCH1*/Δ*dhch1::PAC)* heterozygote described in Fig. 2A; 10/10 transfectants analyzed had the predicted genotype depicted. These DHCH1/PAC/+FTL lines (*DHCH1*/Δ*dhch1::PAC* [pXNG4-*FTL*]) were then transfected with the *HYG DHCH1* targeting fragment as described in the legend to Fig. 3A; of the 12 clonal lines tested, all showed the planned replacement of the chromosomal DHCH1 locus and were thus dhch1^{-}/+FTL (Δ*dhch1::HYG DHCH1*/Δ*dhch1::PAC* [pXNG4-FTL]).

B – PCR tests showing loss of the chromosomal *DHCH1*. This was performed as described in the legend to Fig. 3B. NC, negative control lacking DNA; WT, WT DNA; pXNG-DHCH1, WT transfected with pXNG-*DHCH1*. Pac4, Pac5, and Hyg7 are three different lines showing an absence of the predicted chromosomal *DHCH1* PCR product (shown by arrow). **C.** Western blot showing loss of DHCH1 expression in *dhch1*$^{-}$/+*FTL* transfectants. The lines are as described in part B, extracts from which were probed with polyclonal rabbit antisera against DHCH1 (1:3,000) (Vickers et al., 2008).

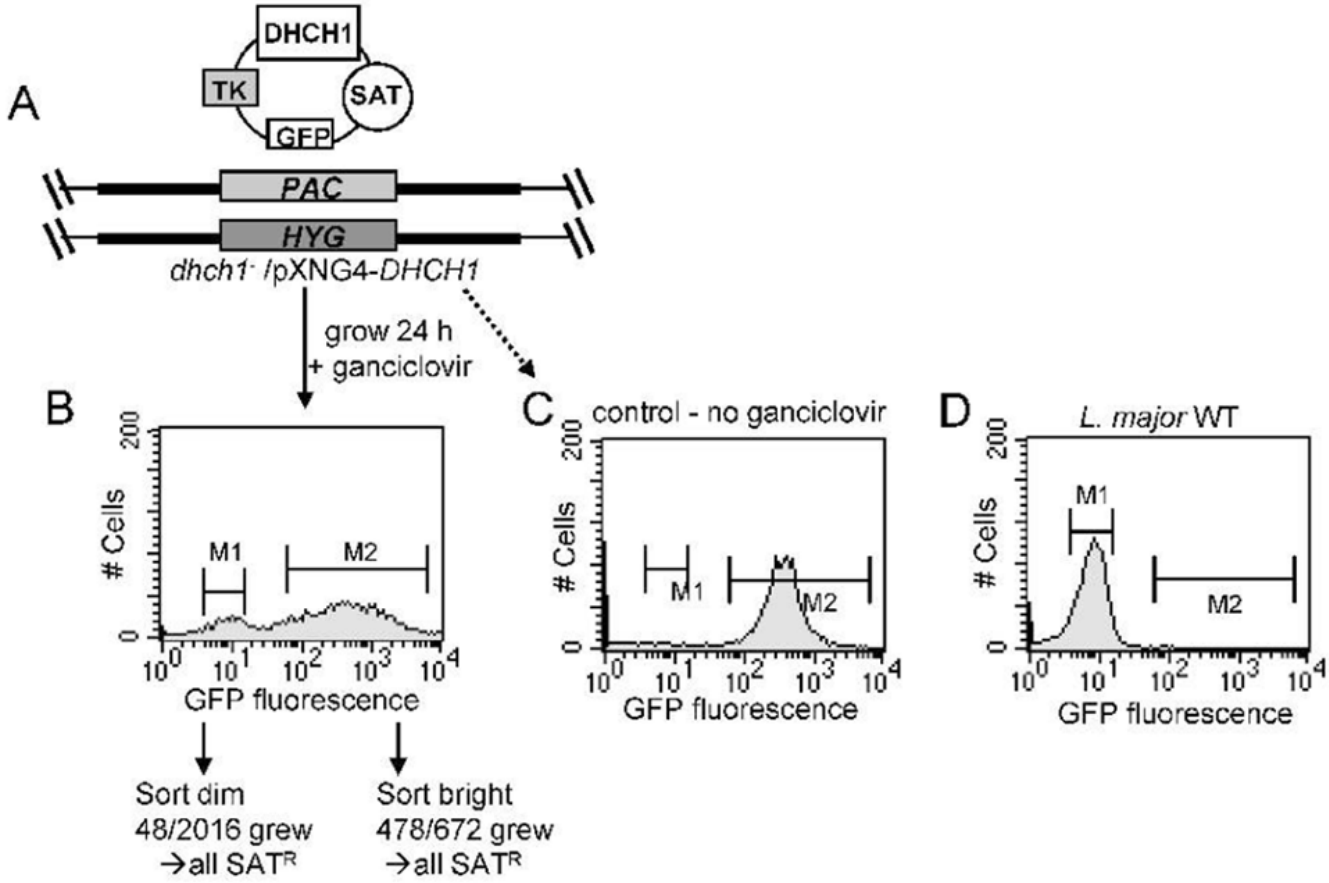

FIG. 5. Plasmid segregational tests of *DHCH1* essentiality

A. Starting *dhch*1⁻/+pXNG4-*DHCH1* transfectant. The structure of one of the lines generated as described in Fig. 3A is shown, additionally showing the TK (ganciclovir sensitivity), GFP and SAT resistance markers encoded on the pXNG4-*DHCH1* vector. B. Forced loss of pXNG4-DHCH1 by negative selection and sorting. The line depicted in Panel A was grown for 24 hr in the presence of ganciclovir but in the absence of nourseothricin. Fluorescent (bright) and non-fluorescent (dim) parasites were assigned using the M1 and M2 gates shown, and single cells were sorted into 96-well plates containing M199 medium and/or various supplements (see methods). With 'bright' cells, 478/672 (71 %) of the wells yielded growth. In contrast, only 48/2016 of wells inoculated with 'dim' cells (2.4 %) showed growth; all of these continued to grow in the presence of nourseothricin (SAT resistance) establishing retention of the pXNG-*DHCH1* episome. **C.** Retention of pXNG4-*DHCH1* in the absence of ganciclovir selection. The line shown in panel A was grown 24 hr without ganciclovir prior to GFP flow cytometry. D. WT *L. major* subjected to GFP flow cytometry.

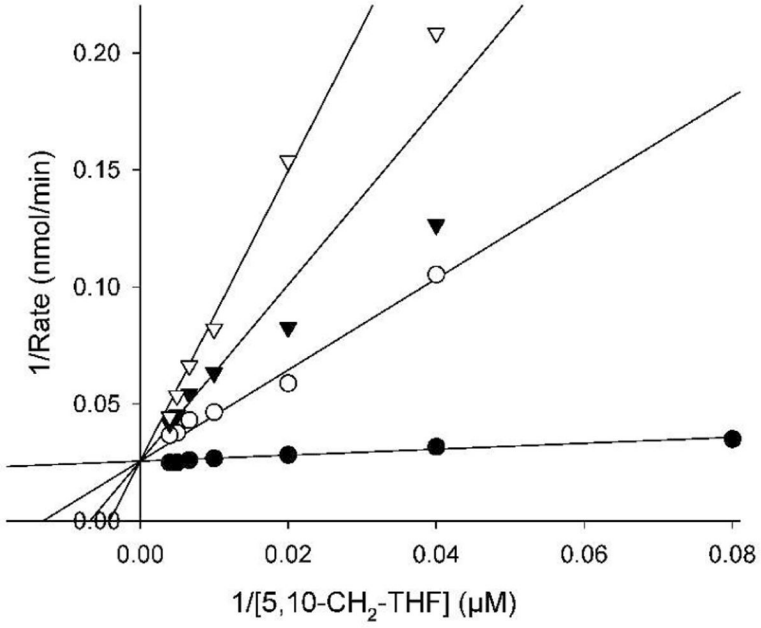

FIG. 6. Inhibition of *L. major* DHCH1 by 5,10-CO-THF

The dehydrogenase activity of purified recombinant DHCH1 (1.3 μg was assayed in the presence of 1 mM NADP+ and varying concentrations of 5,10-CH_2-THF substrate (12.5 to 250 μM) in the presence of no (●), 1.5 μM (○), 3 μM (▼) or 5 μM (▽) inhibitor. The lines show a global fit of these data to a simple competitive mode of inhibition.

TABLE 1

Kinetic constants of recombinant *Leishmania major* DHCH1

Substrate	K_m (app) (μM)	k_{cat} [c] (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1}) × 10^5
5,10-CH_2-THF [a]	120 ± 10	14 ± 1	1.2
$NADP^+$ [b]	38 ± 6	10 ± 1	2.6

[a] Measured with 1 mM $NADP^+$ as the fixed substrate.

[b] Measured with 250 μM 5,10-CH_2-THF as the fixed substrate.

[c] Calculated assuming one active site per polypeptide.

[d] All values given with the standard error of fit.

TABLE 2

Effects of 5,10-CO-THF and antifolates compound on growth of FV1 promastigote lines grown in M199 culture medium

***L. major* FV1 Lines**	**5,10-CO-THF EC_{50} (μM)**	**MTX EC_{50} (nM)**	**Compound 34 EC_{50} (nM)**	**Compound 70 EC_{50} (nM)**
WT	1.1 ± 0.04 *	36 ± 1	680 ± 90	440 ± 50
dhch1^{-}/pXNG4-*DHCH1*	4.5 ± 0.5	41 ± 2	760 ± 80	400 ± 50
dhch1^{-}/pXNG4-*FTL*	3.3 ± 0.3	40 ± 2	540 ± 60	420 ± 40
WT/pXNG4-*DHCH1*	5.1 ± 0.8	n.d.	n.d.	n.d.
WT/pXNG4-*FTL*	3.7 ± 0.5	n.d.	n.d.	n.d.

Compound 5,10-CO-THF is ((2S)-2-[[4-[(6aR)-3-amino-1,9-dioxo-5,6,6a,7-tetrahydro-4H- imidazo[3,4-f]pteridin-8-yl]benzoyl]amino]pentanedioic acid); MTX: Methotrexate; Compound 34 is (2,4-diamino-6-(3,4-dichlorophenoxy)-quinazoline); Compound 70 is (2,4-diamino-6-benzyl-5-(3-phenylpropyl)-pyrimidine).

* EC_{50} for 5,10-CO-THF differs significantly between WT and all other lines tested ($p < 0.0001$ to $p < 0.0003$). n.d. not determined.