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Folate metabolic pathways in *Leishmania*

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Abstract

Trypanosomatid parasitic protozoans of the genus *Leishmania* are autotrophic for both folate and unconjugated pteridines. *Leishmania* salvage these metabolites from their mammalian hosts and insect vectors through multiple transporters. Within the parasite, folates are reduced by a bifunctional DHFR (dihydrofolate reductase)-TS (thymidylate synthase) and by a novel PTR1 (pteridine reductase 1), which reduces both folates and unconjugated pteridines. PTR1 can act as a metabolic bypass of DHFR inhibition, reducing the effectiveness of existing antifolate drugs. *Leishmania* possess a reduced set of folate-dependent metabolic reactions and can salvage many of the key products of folate metabolism from their hosts. For example, they lack purine synthesis, which normally requires 10-formyltetrahydrofolate, and instead rely on a network of purine salvage enzymes. *Leishmania* elaborate at least three pathways for the synthesis of the key metabolite 5,10-methylene-tetrahydrofolate, required for the synthesis of thymidylate, and for 10-formyltetrahydrofolate, whose presumptive function is for methionyl-tRNA^{Met} formylation required for mitochondrial protein synthesis. Genetic studies have shown that the synthesis of methionine using 5-methyltetrahydrofolate is dispensable, as is the activity of the glycine cleavage complex, probably due to redundancy with serine hydroxymethyltransferase. Although not always essential, the loss of several folate metabolic enzymes results in attenuation or loss of virulence in animal models, and a null *DHFR-TS* mutant has been used to induce protective immunity. The folate metabolic pathway provides numerous opportunities for targeted chemotherapy, with strong potential for ‘repurposing’ of compounds developed originally for treatment of human cancers or other infectious agents.

Introduction

Folic acid and related pteridines are essential cofactors in all forms of life, playing critical roles in key metabolic interconversions involving or leading to the transfer of C1 (one-carbon) units [1]. Folic acid is a ‘conjugated’ pteridine, where the pteridine ring is linked to pABA (*p*-aminobenzoic acid), which is in turn glutamylated (Figure 1). Within cells, folate is further polyglutamylated, a modification that aids retention and compartmentalization. Unconjugated pteridines also play important roles in many organisms, although the present chapter will concentrate on the folate pathways. Folates are typically involved in reactions involving the transfer of single-carbon moieties, and these pathways are often referred to collectively as ‘C1 metabolism’.

Pteridine metabolism has long been studied in *Leishmania* and related trypanosomatids. Historically, the activity of biopterin in any organism was revealed by its requirement for growth of the insect trypanosomatid *Crithidia fasciculata*. This bioassay led to the understanding of biopterin’s role as a cofactor for amino acid hydroxylases, followed by

nitric oxide synthase and ether lipid cleavage enzymes [2]. Subsequent studies showed that trypanosomatid protozoans are general pteridine auxotrophs, requiring exogenous pteridines to satisfy their needs for both unconjugated and conjugated pteridines (Figure 1). In contrast, many prokaryotes and many eukaryotes can synthesize both forms. Notably, both the insect and mammalian hosts of trypanosomatid parasites synthesize unconjugated pteridines including biopterin *de novo*, but must acquire folate from the gut (Figure 1).

The extent to which *Leishmania* can interconvert unconjugated pteridines into folates is a matter of some debate. *Leishmania* can generally grow when provided with biopterin alone. However, the presence of trace levels of folate from serum or other medium components and *Leishmania*'s high-affinity FTs (folate transporters) system compromises inferences as to whether *Leishmania* uses biopterin to synthesize folate [3]. Although *Leishmania donovani* can convert radiolabelled biopterin into folic acid [4], *Leishmania* cannot incorporate radiolabelled pABA [5], and genes homologous with those of other species carrying out such interconversions are not apparent in *Leishmania* genomes. Thus the contribution of *de novo* synthesis is unknown. Regardless, it is clear that African trypanosomes and the species of *Leishmania* studied have independent requirements for biopterin and folate.

Historically, knowledge of the folate metabolic pathway was driven by the discoveries of researchers such as George Hitchings [6] that compounds such as trimethoprim or methotrexate inhibit folate metabolism, providing key tools for subsequent dissection of folate metabolic pathways. Some decades later, the use of folate-pathway inhibitors has proven similarly important in probing not only the role of these pathways in normal *Leishmania* metabolism, but also their role in drug resistance. In particular, studies of drug-induced gene amplification have been critical in establishing tools for *Leishmania* molecular genetics [7,8].

Current research encompasses the characterization and role(s) of specific folate-pathway enzymes in metabolism, in virulence, in resistance to anti-folates and as the targets for new drugs. In the present chapter we will take a 'cofactor's eye' view of *Leishmania* metabolism and interconversions of folates, and highlight differences with other trypanosomatids *en route*. Two previous reviews provide a good perspective on *Leishmania* pterin and general metabolism [8,9]

Acquisition of folates and pteridines

Leishmania species acquire folates via active transport [10,11], using two different transporters that were first identified by functional studies of methotrexate-resistant mutants. These related proteins belong to the *FBT* (folate–biopterin transporter) superfamily of transporters [8,12,13]. Genetic, and more recently genomic, studies have shown that the *FT* subfamily comprises more than ten genes in most *Leishmania* species, found dispersed on several chromosomes as well as in a cluster that is found on chromosome 10 in *Leishmania major* (Table 1). Functional studies in *L. donovani* and *Leishmania tarentolae* have implicated at least two members of the *FT* family as encoding high-affinity transporters able to mediate uptake of folates and methotrexate, but not unconjugated pteridines [12,13]. In contrast, BT1 (biopterin transporter 1), the sole member of the second *FBT* subfamily, mediates uptake of biopterin and folate, but not methotrexate [12,13].

Interestingly, both the *BT1* locus and the broadly acting *PTR1* (pteridine reductase 1; described below) often undergo spontaneous genetic amplification as extra-chromosomal linear or circular elements; this can also follow selection for antifolate resistance. In contrast, spontaneous modifications of *FT* genes or *DHFR* (dihydrofolate reductase)-*TS* (thymidylate synthase) have not been observed, with the alteration/amplification of these genes being driven only by drug pressure [8]. These findings implicate unconjugated

pteridine metabolic pathways as chemotherapeutic targets, and also suggest that cultured *Leishmania* may be under pteridine limitation, unless provided with exogenous biopterin. As noted earlier, both the insect and mammalian hosts synthesize unconjugated pteridines *de novo*, often at high levels [2], consistent with the idea that *Leishmania* normally lives in a pterin-rich environment.

Although members of the *FBT* family are often annotated in genome databases as pteridine transporters, these assignments should be viewed cautiously, as recently a member of this gene family was shown to mediate the specific transport of SAM (*S*-adenosylmethionine) [14]. The future characterization of the specificity of other members of the *FBT* family should prove very interesting. It seems likely that the presence of multiple pteridine transporters could pose a challenge for attempts to block folate uptake as part of chemotherapy.

Following uptake, folates are typically metabolized further to form folyl-polyglutamates, through the action of FPGS (folylpolyglutamate synthetase) [15]. Polyglutamylation may play two roles, first aiding the retention of folates within cells, and secondly assisting their compartmentalization within the cell – although this role is less well understood. *Leishmania* species possess a single *FPGS* gene, and *FPGS* over- or under-expression in *L. tarentolae* resulted in increased or decreased levels of folylpolyglutamates (with Glu₄₋₆ predominating). This caused corresponding changes in the parasites' resistance to methotrexate, which is less efficiently polyglutamylated by FPGS [16–18]. Efforts to generate FPGS-homozygous replacements were unsuccessful, suggesting that this gene may be essential [16]. De-glutamylating activities have also been reported, although their roles other than contributing to methotrexate resistance have not been explored [18].

Activation of folate to tetrahydrofolate by DHFR and PTR1

The active forms of both folate and biopterin are the reduced tetrahydro-derivatives, which arise in *Leishmania* through the action of DHFR or the alternative pteridine reductase PTR1. In trypanosomatids and other protozoans, as well as in plants, DHFR is encoded as a fusion protein with TS, with which it functions as part of the *de novo* cycle for dTMP synthesis [19] (Figure 2), as discussed below. Functional and structural studies show that trypanosomatid DHFRs generally resemble those of other species. In contrast, structural and enzymatic studies revealed that PTR1 is a member of the SDR (short-chain dehydrogenase/reductase) family [8,20,21]. Interestingly, the SDR family has proven an evolutionary source for the emergence of a number of pteridine metabolic enzymes, including QDPR (quinonoid dihydropteridine reductase) and other novel pteridine reductases in prokaryotes [22].

In laboratory-adapted *Leishmania*, *DHFR-TS* is essential in the absence of exogenous thymidine [23]. *dhfr-ts*-null mutants survive for less than 60 days within the animal host, where salvage of reduced folates and thymidylate appears to be insufficient, rendering parasites unable to cause disease but able to confer good protection from subsequent challenge with virulent organisms [24]. Similarly, *PTR1* is not essential in attenuated laboratory-adapted strains [20,25], nor required for animal virulence there [26]. However, it has proven impossible to ablate *DHFR-TS* in fully virulent *L. major* [27], and similarly it has proven difficult to ablate *PTR1* in virulent *L. major* without a concomitant irreversible attenuation of animal infectivity (D. A. Scott and S.M. Beverley, unpublished work). These studies challenge the notion that we fully understand the role of the pteridine metabolites in virulent *Leishmania* relevant to human disease, and hint at new and/or unexplored roles for pteridines.

In trypanosomes, PTR1 appears to be essential [28]. This may arise from the finding that trypanosomes lack *QDPR* genes, and must rely upon the QDPR activity of PTR1, whereas

Leishmania bear an array of active *QDPR* genes. Thus trypanosomes may use PTR1 to 'bypass' *QDPR* in a manner not employed by *Leishmania*.

Unlike many other species, *Leishmania* DHFR is unable to reduce unconjugated pteridines, which can only be activated by PTR1 [21] (Figure 2). However, both enzymes are able to reduce folate through to H₄F (also called THF; tetrahydrofolate). Thus trypanosomatid PTR1s provide an alternative metabolic bypass for H₄F synthesis. Although enzymatic and genetic studies have established that DHFR is responsible for the bulk of H₄F synthetic activity in cells [21], the PTR1 bypass acts to greatly mitigate antifolate susceptibility [20,21,25]. This arises primarily from the fact that PTR1 is much less sensitive to conventional antifolates (more than 1000-fold less so for the classic inhibitor methotrexate [29]).

The PTR1 bypass of DHFR thus accounts for the lack of success of most efforts to develop antifolates against trypanosomatids. This illustrates how understanding mechanisms of drug action and resistance in turn serves to guide future efforts [29,30]. One approach has been to identify agents able to inhibit both DHFR and PTR1; although several have been identified, the enzymes' structural divergence makes this a daunting task. A second approach has focused on targeting the two activities with two separate inhibitors [29–31]. Notably, the essentiality of PTR1 in trypanosomes suggests that PTR1-selective inhibitors may work well in that species, whereas in *Leishmania* joint inhibition may be necessary, due to the retention of *QDPR*. This raises the interesting possibility that anti-*QDPR* inhibitors could be employed productively against *Leishmania*. A number of efforts employing structural and chemical biological approaches targeting these three pteridine reductases are now underway, promising to yield effective anti-trypanosomatid antifolates in the future [31,32].

Methylene-H₄F (5,10-CH₂-tetrahydrofolate): the crossroads of C1-THF metabolism

Once formed, H₄F enters C1 metabolism through the activity of either SHMT (serine hydroxymethyltransferase), which uses serine as a C1 donor to form glycine and methylene-H₄F (Figure 3), or the GCC (glycine cleavage complex), which takes glycine and H₄F to form methylene-H₄F, CO₂ and NH₃ (Figure 3). Thereafter methylene-H₄F plays a central and critical role in C1 metabolism, leading to dTMP synthesis by TS, to methionine synthesis via MTHFR (methylene-H₄F reductase) or to 10-CHO-H₄F (10-formyltetrahydrofolate) synthesis via the action of DHCH (methylene-H₄F dehydrogenase/methenyl-H₄F cyclohydrolase).

SHMT

SHMT is encoded by two different genes in *Leishmania*, which encode separate proteins targeted to the cytosol or mitochondrion [33] (Figure 3 and Table 1). This arrangement is seen in other eukaryotes as well and provides some opportunity for both functional redundancy and specialization. Both *SHMT* mRNAs were up-regulated in amastigotes (4-fold), and were modulated to various extents by exogenous glycine, serine or folates [33].

A second reaction mediated by SHMT involves the synthesis of 5-CHO-H₄F, the significance of which is enigmatic, since it inhibits several folate-dependent enzymes and has no known unique metabolic function [34]. To prevent its accumulation and potential toxicity, organisms including *Leishmania* (Table 1) express FCL (5-CHO-H₄F cycloligase), which uses ATP to transform 5-CHO-H₄F into 5,10-methenyl-H₄F, one of the intermediate substrates of DHCH (see below). Unlike the two *SHMT* genes whose proteins are targeted to different compartments, *Leishmania* has only a single identified *FCL*, which lacks a

recognizable mitochondrial-targeting sequence. The predicted cytosolic localization would place it in the same compartment as DHCH1, which is required for further metabolism of the 5,10-methenyl-H₄F product of FCL (Figure 3).

The glycine cleavage complex (GCC)

The GCC is typically found in the mitochondrion as a loose multi-enzyme complex whose members do not occur stoichiometrically. *Leishmania* and trypanosomes bear clear orthologues of the GCC P-protein encoded by *GCV-P* (which mediates the first decarboxylation step), the folate-binding aminomethyl transferase GCC T-protein (encoded by *GCV-T*), the dihydrolipoamide carrier GCC H-protein (encoded by *GCV-H*) and DHLDH (dihydrolipoamide dehydrogenase) (Table 1). DHLDH plays important roles for several other mitochondrial proteins, and the trypanosomatid genomes predict several additional DHLDH-like proteins whose metabolic activities or roles have not been established [35,36] (Table 1). Like *SHMT*, GCC mRNAs are up-regulated in amastigotes, under the control of their 3' untranslated regions [36]. Null mutants lacking *GCV-P* (*gcvp*⁻) lacked GCC activity as expected [35], but were viable when cultured *in vitro*. *gcvp*⁻ mutants were more sensitive to methotrexate, and their growth was reduced by serine limitation, but they were able to infect mice, albeit with some attenuation. This suggests that *Leishmania* cannot scavenge sufficient serine *in vivo* to enable SHMT to fully compensate for the loss of GCC activity.

The importance of methylene-H₄F to *Leishmania* is underscored by the occurrence of three potentially redundant systems for its synthesis, and thus future studies will need to probe the relative contributions of all three enzymes to viability, drug susceptibility and virulence. Curiously, the methylene-H₄F pathways appear more circumscribed in trypanosomes, with a complete absence of SHMT in African trypanosomes. Thus, in *T. brucei*, one could predict a more prominent role for GCC activity, and this may be a particularly attractive area for future studies, both functionally and as a potential 'weakest link' for chemotherapy.

TS

Although most folate-dependent metabolites can be salvaged reasonably well from the host, thymidine typically occurs at levels insufficient to meet the demands for parasite viability. The sole metabolic route towards thymidine synthesis in eukaryotes is TS [37]. Although in most creatures DHFR and TS are both monofunctional, in trypanosomatids and other protozoans, and in plants, TS is fused to the C-terminus of DHFR [38]. This may reflect a unique aspect of the TS enzymatic reaction, which takes dUMP and methylene-H₄F to form dTMP and H₂F (dihydrofolate), differing from all other known C1-folate-utilizing enzymes in oxidizing H₄F. Co-joining of these two activities could help to ensure a functional co-regulation necessary to avoid depletion of H₄F, and/or cellular co-localization required to drive dTMP synthesis. Interestingly, DHFR-TS typically bears a short N-terminal extension ahead of the DHFR domain, which may act as a weak organellar-targeting signal, thus accounting for the presence of DHFR-TS in both cytosolic and mitochondrial compartments [39,40] (Figure 3).

The importance of TS activity in *Leishmania* and trypanosomes has been clearly shown by genetic ablation studies [23,41], and *Leishmania dhfr-ts*⁻ mutants cannot survive long within the mammalian host unless exceptional measures are taken to provide high and near-toxic levels of thymidine [24]. Despite genetic validation as a drug target in both trypanosomes and *Leishmania*, remarkably little effort has gone into developing species-selective inhibitors against TS. However, a few inhibitors selective for *Leishmania* TS over DHFR exist [42] and species-selective TS inhibitors have been reported [43]. For the bifunctional DHFR-TS, structural studies have revealed differences potentially suitable for inhibitor design [44], including the N-terminal leader peptide and the 'bridge' between DHFR-TS

domains [45]. The close proximity of the DHFR and TS catalytic sites raises the possibility of 'bridged' inhibitors able to synergistically thymidylate synthesis at two successive steps.

Synthesis of methionine requires methyl-H₄F: a non-essential pathway?

The metabolic demands and flux through the methionine biosynthetic pathways are substantial in trypanosomatids. Methionine plays critical roles, as an essential amino acid and for formation of SAM, which is required for essential methylation reactions and the synthesis of polyamines. Polyamines are critically important in trypanosomatids for the synthesis of trypanothione, the major cellular thiol [46]. Methionine can be salvaged or synthesized by methionine synthase, which uses methyl-H₄F and homocysteine to produce methionine and H₄F (Figure 3). Homocysteine can be taken up or arise through the activity of SAM-dependent methylations. Two predicted MetS (methionyl-tRNA synthetase) genes are found in the *L. major* genome, one showing similarity to the cobalamin-dependent MetS and one the cobalamin-independent MetS, which may have been acquired by lateral gene transfer from prokaryotes (Table 1). Neither the function nor localization of these two proteins has been explored. In other eukaryotes MetS is located in the cytosol, and neither predicted *Leishmania* protein bears a clear mitochondrial-targeting sequence.

MetS proteins require a source of 5-CH₃-H₄F (5-methyltetrahydrofolate), which could be acquired by salvage as this is the predominant reduced folate in mammalian serum. Methyl-H₄F arises intracellularly from methylene-H₄F through the action of MTHFR. Unlike most species, *Leishmania* MTHFR utilizes either NADH or NADPH and lacks allosteric regulation [47]. Whereas under conditions of methionine limitation *methfr*-null mutants grew less well, in standard culture medium they grew normally and showed wild-type infectivity in susceptible mice [47]. *methfr*-null mutants were unable to use homocysteine in place of methionine and lacked detectable methyl-H₄F, showing that no alternative path for methyl-H₄F synthesis exists in *Leishmania*.

Thus under the conditions tested *in vitro* or *in vivo*, provision of methionine via *MTHFR*, and presumably by either or both MetS proteins, is not limiting. African trypanosomes have gone a step further and have retained neither *METS* nor *MTHFR*.

10-CHO-THF: the second critical form of C1-folate

10-CHO-H₄F is generated either by the ATP-dependent ligation of formate and H₄F, catalysed by FTL (formyltetrahydrofolate ligase), or the oxidation of 5,10-CH₂-H₄F to 5,10-CH=H₄F (5,10-methenyltetrahydrofolate), catalysed by a methylene-tetrahydrofolate DH (dehydrogenase) and then hydrolysis of methenyltetrahydrofolate by a CH (cyclohydrolase). These activities are found in various configurations, ranging from monofunctional FTLs, bifunctional DHCHs or even, in organisms such as yeast, all three activities combined in a single multifunctional polypeptide termed the C1-synthase [48]. Adding to the complexity, in eukaryotes, different isoforms of these enzymes can be found in either the mitochondrion or the cytosol.

In *L. major*, 10-CHO-H₄F is produced by a cytosolic monofunctional FTL and a cytosolic bifunctional DHCH1 [49]. *L. major* also encodes a DHCH pseudogene (*pseDHCH2*) [49] (Table 1), but *Leishmania braziliensis* and trypanosomes lack a *DHCH2*-related gene. Interestingly, the genomes of *L. donovani* and *Leishmania mexicana* suggest the presence of an intact *DHCH2*, bearing an N-terminal extension resembling mitochondrial-targeting sequences, and heterologously expressed *Leishmania infantum* *DHCH2* was localized to the mitochondrion of *L. major* [49]. Attempts to demonstrate enzymatic activity of *DHCH2* *in vitro* or *in vivo* were unsuccessful. Thus in *L. major* and perhaps all trypanosomatids, 10-CHO-H₄F synthetic activities occur exclusively in the cytosol.

Known roles of 10-CHO-H₄F include the *de novo* synthesis of purines and the formylation of the mitochondrial initiator methionyl-tRNA^{Met} (fMet-tRNA^{Met}). However *Leishmania* and other trypanosomatids (other than those bearing endosymbionts) lack the *de novo* purine synthetic pathway. Trypanosomatids maintain pathways involved in the synthesis of formylated initiator tRNA^{Met}, as deduced from genome sequencing, which predicts the presence of the formyl transferase, the mitochondrial IF2 (translation-initiation factor 2) that binds formylated initiator tRNA^{Met}, and peptide deformylases (Table 1 and Figure 3), and synthesize fMet-tRNA^{Met} [50]. Curiously, trypanosomes formylate the eukaryotic-type elongator tRNA^{Met-e} after import into the mitochondrion for initiation of protein synthesis, rather than the tRNA^{Met-i} used for this purpose by other species [50]. There is considerable debate about the requirement for initiator Met-tRNA^{Met} formylation, with genetic studies in both prokaryotes and eukaryotes yielding divergent conclusions – often within the same species [51]. RNAi (RNA interference) studies in trypanosomes showed little phenotype when targeting solely *FMT*, but strong growth inhibition when both *FMT* and *IF2* were targeted simultaneously [52].

In mammals and yeast, metabolic labelling suggests that the formation of mitochondrial methylene-H₄F through the activities of *FTL* and *DHCH* is a major source of active C1 units [53]. A *Leishmania ftl*-null mutant had no detectable phenotype *in vitro* or *in vivo* and trypanosomes lacked *FTL*, implying that methylene-H₄F arising from serine (SHMT) or glycine (GCC) is sufficient. When the activity of these pathways was reduced through serine or glycine limitation, growth of the *L. major ftl*-null mutant was inhibited, suggesting that formate can be incorporated into methylene-H₄F.

In *L. major*, *DHCH1* is essential, despite the continued presence of *FTL*, and pharmacological inhibition by a substrate/transition-state analogue suggests that this enzyme has potential as a drug target [54]. Loss of *DHCH1* could be rescued, however, by overexpression of *FTL*, establishing definitely by ‘metabolite complementation’ that 10-CHO-THF is the essential metabolite. Thus it seems likely that mitochondrial Met-tRNA^{Met} formylation accounts for the essentiality of 10-CHO-H₄F. However, novel roles cannot be ruled out, neither can a role for the *DHCH* intermediate 5–10-methenyl-H₄F be excluded. If required for mitochondrial Met-tRNA^{Met} formylation, there must be some pathway for 10-CHO-H₄F transport from the cytosolic site of synthesis into the mitochondrion in both *Leishmania* and trypanosomes.

Novel roles for folates in *Leishmania*: YGFZ and iron–sulfur cluster protein function

As noted earlier, there is ample reason to believe that novel roles for pteridines remain to be discovered in trypanosomatids. One recent example involves the mitochondrial protein YGFZ [55]. This protein shows structural homology with GCV-T, which binds folate as part of the aminotransferase step of the glycine cleavage reaction. Modelling studies suggested that EcYgfZ (*Escherichia coli* YgfZ) bears a folate-binding site, and recombinant EcYgfZ bound folates, albeit with a modest *K_m* [55]. In *E. coli* and yeast, loss of YGFZ (*Iba57* in yeast notation) caused defects in a subset of iron–sulfur enzymes [56], and *E. coli* ygfZ mutants show hypersensitivity to oxidative stress, phenotypes that are mimicked in $\Delta folE$ mutants lacking both pterins and folates, or in $\Delta folP$ mutants that lack folates. Notably *Leishmania* and other species’ YGFZs were able to complement *E. coli* ygfZ[−] mutants, and efforts to generate *Leishmania* ygfZ[−] mutants are underway.

The mechanism of this novel folate-dependent protein is not understood, and neither is it understood why only a subset of iron–sulfur cluster proteins are affected in *E. coli* or yeast mutants. Hypotheses include the transfer of reducing equivalents to an iron–sulfur cluster

during assembly, or the repair of damaged clusters by the removal of C1 adducts such as formaldehyde and their subsequent transfer to H₄F [55].

Conclusion and future studies

Folate metabolism has provided rich grounds for discovery in trypanosomatid parasites. The formation of reduced folate via DHFR or PTR1, the formation of methylene-H₄F needed for thymidylate synthesis and the formation of 10-CHO-H₄F for mitochondrial protein synthesis are all essential processes suitable for chemotherapeutic attack, and indeed efforts on several of these are well advanced. Key remaining questions include a better understanding of how folate metabolism is compartmentalized and dynamically organized. Evolutionary comparisons among trypanosomatids raise a number of important questions; although folate pathways are largely conserved, significant differences are evident. Unlike *Leishmania*, African trypanosomes seem to rely on just one pathway (GCC) for the formation of methylene-H₄F, rendering them particularly susceptible to its inhibition. Why *Leishmania* has retained the MTHFR/MetS pathways, whereas trypanosomes survive perfectly well without them, is unknown. Proof that the requirement for 10-CHO-H₄F rests on a need for this in mitochondrial protein synthesis should emerge in the near future. Lastly, it seems very likely that novel roles for folates and pterins will soon surface in trypanosomatids, as exemplified by the recent link of the folate-binding protein YgfZ with iron–sulfur cluster protein metabolism, and the as yet inexplicable differences in the requirements for PTR1 and DHFR in laboratory-adapted compared with fully virulent *Leishmania*.

Summary

- *Leishmania* are folate auxotrophs and salvage both folic acid and multiple metabolites that are usually produced in folate metabolism.
- *Leishmania* have two pteridine reductases, a bifunctional DHFR-TS and a novel PTR1 that can reduce both unconjugated pteridines and folate.
- Methylene-H₄F is the key C1 intermediate in *Leishmania* and is required for both the synthesis of thymidylate and further metabolism to 10-CHO-H₄F.
- SHMT and the GCC are the main paths for methylene-H₄F biosynthesis in *Leishmania* and appear to be functionally redundant.
- 10-CHO-H₄F is essential for parasite survival and, since *Leishmania* are purine auxotrophs, this implies that the formylation of the initiator methionyl-tRNA in the *Leishmania* mitochondrion is also essential, as this is the sole remaining function known for this folate.
- YGFZ provides a link between folate metabolism and iron–sulfur cluster function, although its mechanism of action is unknown.

References

1. Fox JT, Stover PJ. Folate-mediated one-carbon metabolism. *Vitam. Horm.* 2008; 79:1–44. [PubMed: 18804690]
2. Kaufman, S. Tetrahydrobiopterin: Basic Biochemistry and Role in Human Disease. Baltimore: Johns Hopkins University Press; 1997.
3. Petrillo-Peixoto M, Beverley SM. *In vitro* activity of sulfonamides and sulfones against *Leishmania major* promastigotes. *Antimicrob. Agents Chemother.* 1987; 31:1575–1578. [PubMed: 3435106]
4. Beck JT, Ullman B. Biopterin conversion to reduced folates by *Leishmania donovani* promastigotes. *Mol. Biochem. Parasitol.* 1991; 49:21–28. [PubMed: 1775157]

5. Kovacs JA, Allegra CJ, Beaver J, Boarman D, Lewis M, Parrillo JE, Chabner B, Masur H. Characterization of *de novo* folate synthesis in *Pneumocystis carinii* and *Toxoplasma gondii* : potential for screening therapeutic agents. *J. Infect. Dis.* 1989; 160:312–320. [PubMed: 2527275]
6. Hitchings GH Jr. Nobel lecture in physiology or medicine – 1989. Selective inhibitors of dihydrofolate reductase. *In Vitro Cell. Dev. Biol.* 1988; 25:303–310. [PubMed: 2654121]
7. Beverley SM. Gene amplification in *Leishmania*. *Annu. Rev. Microbiol.* 1991; 45:417–444. [PubMed: 1741620]
8. Ouellette M, Drummelsmith J, El-Fadili A, Kundig C, Richard D, Roy G. Pterin transport and metabolism in *Leishmania* and related trypanosomatid parasites. *Int. J. Parasitol.* 2002; 32:385–398. [PubMed: 11849635]
9. Oppenheimer FR, Coombs GH. Metabolism of *Leishmania*: proven and predicted. *Trends Parasitol.* 2007; 23:149–158. [PubMed: 17320480]
10. Ellenberger TE, Beverley SM. Biochemistry and regulation of folate and methotrexate transport in *Leishmania major*. *J. Biol. Chem.* 1987; 262:10053–10058. [PubMed: 2886498]
11. Kaur K, Coons T, Emmett K, Ullman B. Methotrexate-resistant *Leishmania donovani* genetically deficient in the folate-methotrexate transporter. *J. Biol. Chem.* 1988; 263:7020–7028. [PubMed: 3366764]
12. Kundig C, Haimeur A, Legare D, Papadopoulou B, Ouellette M. Increased transport of pteridines compensates for mutations in the high affinity folate transporter and contributes to methotrexate resistance in the protozoan parasite *Leishmania tarentolae*. *EMBO J.* 1999; 18:2342–2351. [PubMed: 10228149]
13. Lemley C, Yan S, Dole VS, Madhubala R, Cunningham ML, Beverley SM, Myler PJ, Stuart KD. The *Leishmania donovani* LD1 locus gene *ORFG* encodes a biopterin transporter (BT1). *Mol. Biochem. Parasitol.* 1999; 104:93–105. [PubMed: 10589984]
14. Dridi L, Ahmed-Ouameur A, Ouellette M. The high affinity *S*-adenosylmethionine plasma membrane transporter of *Leishmania* is a member of the folate biopterin transporter (FBT) family. *J. Biol. Chem.* 2010; 285:19767–19775. [PubMed: 20406813]
15. Santi DV, Nolan P, Shane B. Folylpolyglutamates in *Leishmania major*. *Biochem. Biophys. Res. Commun.* 1987; 146:1089–1092. [PubMed: 3619916]
16. Fadili AE, Kundig C, Ouellette M. Characterization of the folylpolyglutamate synthetase gene and polyglutamylase of folates in the protozoan parasite *Leishmania*. *Mol. Biochem. Parasitol.* 2002; 124:63–71. [PubMed: 12387851]
17. El-Fadili A, Richard D, Kundig C, Ouellette M. Effect of polyglutamylase of methotrexate on its accumulation and the development of resistance in the protozoan parasite *Leishmania*. *Biochem. Pharmacol.* 2003; 66:999–1008. [PubMed: 12963486]
18. Ellenberger TE, Wright JE, Rosowsky A, Beverley SM. Wild-type and drug-resistant *Leishmania major* hydrolyze methotrexate to *N*-10-methyl-4-deoxy-4-aminopteroate without accumulation of methotrexate polyglutamates. *J. Biol. Chem.* 1989; 264:15960–15966. [PubMed: 2476435]
19. Beverley SM, Ellenberger TE, Cordingley JS. Primary structure of the gene encoding the bifunctional dihydrofolate reductase-thymidylate synthase of *Leishmania major*. *Proc. Natl. Acad. Sci. U.S.A.* 1986; 83:2584–2588. [PubMed: 3458220]
20. Bello AR, Nare B, Freedman D, Hardy L, Beverley SM. PTR1: a reductase mediating salvage of oxidized pteridines and methotrexate resistance in the protozoan parasite *Leishmania major*. *Proc. Natl. Acad. Sci. U.S.A.* 1994; 91:11442–11446. [PubMed: 7972081]
21. Nare B, Hardy LW, Beverley SM. The roles of pteridine reductase 1 and dihydrofolate reductase-thymidylate synthase in pteridine metabolism in the protozoan parasite *Leishmania major*. *J. Biol. Chem.* 1997; 272:13883–13891. [PubMed: 9153248]
22. Varughese KI, Xuong NH, Kiefer PM, Matthews DA, Whiteley JM. Structural and mechanistic characteristics of dihydropteridine reductase: a member of the Tyr-(Xaa)₃-Lys-containing family of reductases and dehydrogenases. *Proc. Natl. Acad. Sci. U.S.A.* 1994; 91:5582–5586. [PubMed: 8202530]
23. Cruz A, Beverley SM. Gene replacement in parasitic protozoa. *Nature.* 1990; 348:171–173. [PubMed: 2234081]

24. Titus RG, Gueiros-Filho FJ, de Freitas LA, Beverley SM. Development of a safe live *Leishmania* vaccine line by gene replacement. *Proc. Natl. Acad. Sci. U.S.A.* 1995; 92:10267–10271. [PubMed: 7479765]
25. Papadopoulos B, Roy G, Mourad W, Leblanc E, Ouellette M. Changes in folate and pterin metabolism after disruption of the *Leishmania* H locus short chain dehydrogenase gene. *J. Biol. Chem.* 1994; 269:7310–7315. [PubMed: 8125946]
26. Cunningham ML, Titus RG, Turco SJ, Beverley SM. Regulation of differentiation to the infective stage of the protozoan parasite *Leishmania major* by tetrahydrobiopterin. *Science.* 2001; 292:285–287. [PubMed: 11303103]
27. Cruz AK, Titus R, Beverley SM. Plasticity in chromosome number and testing of essential genes in *Leishmania* by targeting. *Proc. Natl. Acad. Sci. U.S.A.* 1993; 90:1599–1603. [PubMed: 8381972]
28. Ong HB, Sienkiewicz N, Wyllie S, Fairlamb AH. Dissecting the metabolic roles of pteridine reductase 1 in *Trypanosoma brucei* and *Leishmania major*. *J. Biol. Chem.* 2011; 286:10429–10438. [PubMed: 21239486]
29. Nare B, Luba J, Hardy LW, Beverley S. New approaches to *Leishmania* chemotherapy: pteridine reductase 1 (PTR1) as a target and modulator of antifolate sensitivity. *Parasitology.* 1997; 114:S101–S110. [PubMed: 9309772]
30. Hardy LW, Matthews W, Nare B, Beverley SM. Biochemical and genetic tests for inhibitors of *Leishmania* pteridine pathways. *Exp. Parasitol.* 1997; 87:157–169. [PubMed: 9371081]
31. Cavazzuti A, Paglietti G, Hunter WN, Gamarro F, Piras S, Loriga M, Allecca S, Corona P, McLuskey K, Tulloch L, et al. Discovery of potent pteridine reductase inhibitors to guide antiparasite drug development. *Proc. Natl. Acad. Sci. U.S.A.* 2008; 105:1448–1453. [PubMed: 18245389]
32. Gourley DG, Schuttelkopf AW, Leonard GA, Luba J, Hardy LW, Beverley SM, Hunter WN. Pteridine reductase mechanism correlates pterin metabolism with drug resistance in trypanosomatid parasites. *Nat. Struct. Biol.* 2001; 8:521–525. [PubMed: 11373620]
33. Gagnon D, Foucher A, Girard I, Ouellette M. Stage specific gene expression and cellular localization of two isoforms of the serine hydroxymethyltransferase in the protozoan parasite *Leishmania*. *Mol. Biochem. Parasitol.* 2006; 150:63–71. [PubMed: 16876889]
34. Stover P, Schirch V. The metabolic role of leucovorin. *Trends Biochem. Sci.* 1993; 18:102–106. [PubMed: 8480361]
35. Scott DA, Hickerson SM, Vickers TJ, Beverley SM. The role of the mitochondrial glycine cleavage complex in the metabolism and virulence of the protozoan parasite *Leishmania major*. *J. Biol. Chem.* 2008; 283:155–165. [PubMed: 17981801]
36. Muller M, Papadopoulos B. Stage-specific expression of the glycine cleavage complex subunits in *Leishmania infantum*. *Mol. Biochem. Parasitol.* 2010; 170:17–27. [PubMed: 19961881]
37. Carreras CW, Santi DV. The catalytic mechanism and structure of thymidylate synthase. *Annu. Rev. Biochem.* 1995; 64:721–762. [PubMed: 7574499]
38. Ivanetich KM, Santi DV. Bifunctional thymidylate synthase-dihydrofolate reductase in protozoa. *FASEB J.* 1990; 4:1591–1597. [PubMed: 2180768]
39. Swafford JR, Beverley SM, Kan CC, Caulfield JP. Distribution of dihydrofolate reductase-thymidylate synthase polypeptide in methotrexate resistant and wild type *Leishmania major*: evidence for cytoplasmic distribution despite a hydrophilic leader sequence. *Proc. XII Int. Congr. Electron Microsc.* 1990; 12:610–611.
40. Luo M, Orsi R, Patrucco E, Pancaldi S, Cella R. Multiple transcription start sites of the carrot dihydrofolate reductase-thymidylate synthase gene, and sub-cellular localization of the bifunctional protein. *Plant Mol. Biol.* 1997; 33:709–722. [PubMed: 9132062]
41. Sienkiewicz N, Jaroslawski S, Wyllie S, Fairlamb AH. Chemical and genetic validation of dihydrofolate reductase-thymidylate synthase as a drug target in African trypanosomes. *Mol. Microbiol.* 2008; 69:520–533. [PubMed: 18557814]
42. Garvey EP, Coderre JA, Santi DV. Selection and properties of *Leishmania* resistant to 10-propargyl-5,8-dideazafolate, an inhibitor of thymidylate synthase. *Mol. Biochem. Parasitol.* 1985; 17:79–91. [PubMed: 4058489]

43. Stout TJ, Tondi D, Rinaldi M, Barlocco D, Pecorari P, Santi DV, Kuntz ID, Stroud RM, Shoichet BK, Costi MP. Structure-based design of inhibitors specific for bacterial thymidylate synthase. *Biochemistry*. 1999; 38:1607–1617. [PubMed: 9931028]
44. Knighton DR, Kan CC, Howland E, Janson CA, Hostomska Z, Welsh KM, Matthews DA. Structure of and kinetic channelling in bifunctional dihydrofolate reductase-thymidylate synthase. *Nat. Struct. Biol.* 1994; 1:186–194. [PubMed: 7656037]
45. Atreya CE, Johnson EF, Irwin JJ, Dow A, Massimine KM, Coppens I, Stempluk V, Beverley S, Joiner KA, Shoichet BK, Anderson KS. A molecular docking strategy identifies Eosin B as a non-active site inhibitor of protozoal bifunctional thymidylate synthase-dihydrofolate reductase. *J. Biol. Chem.* 2003; 278:14092–14100. [PubMed: 12556445]
46. Fairlamb AH, Cerami A. Metabolism and functions of trypanothione in the Kinetoplastida. *Annu. Rev. Microbiol.* 1992; 46:695–729. [PubMed: 1444271]
47. Vickers TJ, Orsomando G, de la Garza RD, Scott DA, Kang SO, Hanson AD, Beverley SM. Biochemical and genetic analysis of methylenetetrahydrofolate reductase in *Leishmania* metabolism and virulence. *J. Biol. Chem.* 2006; 281:38150–38158. [PubMed: 17032644]
48. Christensen KE, MacKenzie RE. Mitochondrial one-carbon metabolism is adapted to the specific needs of yeast, plants and mammals. *BioEssays*. 2006; 28:595–605. [PubMed: 16700064]
49. Vickers TJ, Murta SM, Mandell MA, Beverley SM. The enzymes of the 10-formyl-tetrahydrofolate synthetic pathway are found exclusively in the cytosol of the trypanosomatid parasite *Leishmania major*. *Mol. Biochem. Parasitol.* 2009; 166:142–152. [PubMed: 19450731]
50. Tan TH, Bochud-Allemann N, Horn EK, Schneider A. Eukaryotic-type elongator tRNA^{Met} of *Trypanosoma brucei* becomes formylated after import into mitochondria. *Proc. Natl. Acad. Sci. U.S.A.* 2002; 99:1152–1157. [PubMed: 11792845]
51. Li Y, Holmes WB, Appling DR, RajBhandary UL. Initiation of protein synthesis in *Saccharomyces cerevisiae* mitochondria without formylation of the initiator tRNA. *J. Bacteriol.* 2000; 182:2886–2892. [PubMed: 10781559]
52. Charriere F, Tan TH, Schneider A. Mitochondrial initiation factor 2 of *Trypanosoma brucei* binds imported formylated elongator-type tRNA^{Met}. *J. Biol. Chem.* 2005; 280:15659–15665. [PubMed: 15731104]
53. Tibbetts AS, Appling DR. Compartmentalization of mammalian folate-mediated one-carbon metabolism. *Annu. Rev. Nutr.* 2010; 30:57–81. [PubMed: 20645850]
54. Murta SM, Vickers TJ, Scott DA, Beverley SM. Methylene tetrahydrofolate dehydrogenase/cyclohydrolase and the synthesis of 10-CHO-THF are essential in *Leishmania major*. *Mol. Microbiol.* 2009; 71:1386–1401. [PubMed: 19183277]
55. Waller JC, Alvarez S, Naponelli V, Lara-Nunez A, Blaby IK, Da Silva V, Ziemak MJ, Vickers TJ, Beverley SM, Edison AS, et al. A role for tetrahydrofolates in the metabolism of iron–sulfur clusters in all domains of life. *Proc. Natl. Acad. Sci. U.S.A.* 2010; 107:10412–10417. [PubMed: 20489182]
56. Gelling C, Dawes IW, Richhardt N, Lill R, Muhlenhoff U. Mitochondrial Iba57p is required for Fe/S cluster formation on aconitase and activation of radical SAM enzymes. *Mol. Cell. Biol.* 2008; 28:1851–1861. [PubMed: 18086897]

Figure 1. Folates and pterins in *Leishmania* and other organisms

The structures of folic acid (upper left) and biopterin (lower left), a representative unconjugated pterin, are shown. Folates can be further modified by additional γ -glutamyl residues. *Leishmania* (upper right) are auxotrophic for both pteridines and must salvage and activate them, using both DHFR and pteridine reductase to form H₄F and pteridine reductase to form tetrahydrobiopterin (H₄B). In contrast, humans (lower right) can synthesize biopterin starting from GTP, but must salvage folate. Other patterns are seen in evolution; *E. coli* can synthesize both folates and unconjugated pteridines, and the malaria parasite *Plasmodium* can synthesize folates.

Figure 2. Acquisition and activation of pteridines: PTR1 as a metabolic bypass of DHFR

Folate uptake is mediated by several transporters, including FT1 and BT1, and is subsequently reduced to H₂F and H₄F by DHFR. PTR1 also reduces folates and can bypass DHFR inhibition as it is less susceptible to many antifolates, including methotrexate. H₄F is converted into a variety of C1-folate cofactors (Figure 3), including methylene-tetrahydrofolate (CH₂=H₄F), by the action of SHMT or the GCC. Although most C1-folate-utilizing enzymes maintain the H₄F reduction state, TS uniquely causes oxidation back to H₂F. In *Leishmania* and trypanosomes, DHFR and TS are fused into a single bifunctional protein. Biopterin is salvaged by BT1 and reduced by PTR1 to dihydrobiopterin (H₂B) and then H₄B; DHFR is unable to carry out these reductions. H₄B is consumed by phenylalanine hydroxylase (PAH) and probably other reactions as yet unknown. Pterin-dependent hydroxylases yield pterin-4a-carbinolamine, which is recycled by pterin-4a-carbinolamine dehydratase (PCD) to quinoid-dihydrobiopterin (q-H₂B), and then to H₄B through the action of quinoid-dihydrobiopterin reductase (QDPR). This research was originally published in *The Journal of Biological Chemistry*. Nare, B., Hardy, L.W. and Beverley, S.M. The roles of pteridine reductase 1 and dihydrofolate reductase-thymidylate synthase in pteridine metabolism in the protozoan parasite *Leishmania major*. *J. Biol. Chem.* 1997; 272: 13883–13891. © the American Society for Biochemistry and Molecular Biology.

Figure 3. Compartmentalization of folate metabolism in *Leishmania*

Folate metabolites are in light blue and major metabolites produced by folate-dependent enzymes are in yellow. Enzymes are in italicized capitals, with the relevant activity of bifunctional enzymes depicted in a larger font. Enzyme abbreviations and full enzymatic reactions are given in Table 1. Broken arrows depict intracellular transport steps inferred from the requirements and localizations of the known enzymes. The metabolites shown are 5,10-methylene-tetrahydrofolate ($\text{CH}_2=\text{H}_4\text{F}$), 5-methyltetrahydrofolate ($\text{CH}_3\text{-H}_4\text{F}$), 5-formyltetrahydrofolate (5-CHO- H_4F), 10-formyltetrahydrofolate (10-CHO- H_4F), 5,10-methenyltetrahydrofolate ($-\text{CH}=\text{H}_4\text{F}$), methionine (Met), thymidine monophosphate (TMP), glycine (Gly), serine (Ser) and formylated initiator methionyl-tRNA^{Met} (fMet-tRNA). cSHMT, cytosolic SHMT; mSHMT, mitochondrial SHMT.

Folate metabolic pathway genes, enzymes and phenotypes Unless otherwise indicated, essentiality tests cited were performed in *Leishmania major*. B, bioperin; *cSTHM*, cytoplasmic STHM; F, folate; H₂B, dihydrobiopterin; H₄B, tetrahydrobiopterin; mSTHM, mitochondrial STHM; MTX, methotrexate.

Table 1

Enzyme	Gene	EC number	<i>L. major</i> gene IDs	Function	Null mutant phenotype	Reference(s)
Folate transporter family	<i>FT</i> subfamily	–	LmjF06.1260 LmjF06.0310 LmjF10.0020 LmjF10.0360 LmjF10.0380 LmjF10.0385 LmjF10.0390 LmjF10.0400 LmjF10.1310 LmjF19.0920	Folate uptake (+ SAM and/or other substrates?)	MTX resistance, elevated folate requirement	[12,13]
	<i>BTI</i>	–	LmjF35.5150			
Folypolyglutamate synthetase	<i>FPGS</i>	6.3.2.17	LmjF36.2610	Folate polyglutamylation	Essential (<i>L. tarentolae</i>)	[16]
Pteridine reductase 1	<i>PTR1</i>	1.5.1.33	LmjF23.0270	Reduction of B, H ₂ B, F and H ₂ F	Increased metacyclogenesis; essentiality varies with strain background	[20,25]
Dihydrofolate reductase-thymidylate synthase	<i>DHFR-TS</i>	1.5.1.3, 2.1.1.45	LmjF06.0860	Reduction of F and H ₂ F	Essential	[19,23]
Serine <i>trans</i> -hydroxymethyl transferase	<i>cSTHM</i> , <i>mSTHM</i>	2.1.2.1	LmjF14.1320 LmjF28.2370	H ₄ F+serine → methylene-H ₄ F+glycine	Not tested	[33]
Methylenetetrahydrofolate reductase	<i>MTHFR</i>	1.5.1.20	LmjF36.6390	Methylene-H ₄ F → CH ₃ -H ₄ F	Increased methionine requirement	[47]
Methionine synthase	<i>METS</i> (vitamin B ₁₂ -dependent)	2.1.1.13	LmjF07.0090	CH ₃ -H ₄ F+homocysteine → methionine+H ₄ F	Not tested	
	<i>METS</i> (vitamin B ₁₂ -independent)	2.1.1.14	LmjF31.0010			
5-Formyl tetrahydrofolate cycloligase	<i>FCL</i>	6.3.3.2	LmjF23.1045	ATP+5-CHO-H ₄ F → 5,10-methenyl-H ₄ F+ADP+P _i	Not tested	
Glycine cleavage complex				Glycine+H ₄ F → methylene-H ₄ F+CO ₂ +NH ₃	Modest reduction in virulence	[35]
T-protein	<i>GCV-T</i>	2.1.2.10	LmjF36.3800 LmjF36.3810			

Enzyme	Gene	EC number	<i>L. major</i> gene IDs	Function	Null mutant phenotype	Reference(s)
P-protein	<i>GCV-P</i>	1.4.4.2	LmjF26.0030			
H-protein	<i>GCV-H</i>	–	LmjF35.4720			
Dihydrolipoamide dehydrogenase (L-protein)	<i>DLDH</i>	1.8.1.4	LmjF29.1830 LmjF.32.3310			
Formate-tetrahydrofolate ligase	<i>FTL</i>	6.3.4.3	LmjF30.2600	Formate+ATP+H ₄ F → 10-CHO-H ₄ F+ADP+P _i	No phenotype	[49]
Methylenetetrahydrofolate dehydrogenase/methenyl-tetrahydrofolate cyclohydrolase	<i>DHCHI</i>	1.5.1.5, 3.5.4.9	LmjF26.0320	Methylene-H ₄ F → 10-CHO-H ₄ F	Essential	[54]
	<i>pseDHCH2</i>	–	LmjF22.0340	None detected	Pseudogene in <i>L. major</i>	[49]
Formylmethionyl-tRNA transferase	<i>FMT</i>	2.1.2.9	LmjF32.2240	10-CHO-H ₄ F+Met-tRNA ^{Met} → CHO-Met-tRNA ^{Met} +H ₄ F	Likely to be essential (<i>Trypanosoma brucei</i>)	[50]; T. J. Vickers and S. M. Beverley, unpublished work
	<i>YGFZ</i>	–	LmjF24.1740	Required for activity of some Fe-S cluster enzymes	Essential	T. J. Vickers and S. M. Beverley, unpublished work