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Invited Review

Pterin transport and metabolism in *Leishmania* and related trypanosomatid parasites

Marc Ouellette[a,b,*], Jolyne Drummelsmith[a,b], Amal El Fadili[a,b], Christoph Kündig[a,b,1], Dave Richard[a,b], Gaétan Roy[a,b]

[a]*Centre de recherche en Infectiologie du CHUL, 2705, boul. Laurier, Sainte-Foy, QC, Canada GIV 4G2*
[b]*Division de Microbiologie, Faculté de médecine, Université Laval, Laval, Québec, Canada*

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Abstract

The folate metabolic pathway has been exploited successfully for the development of antimicrobial and antineoplasic agents. Inhibitors of this pathway, however, are not useful against *Leishmania* and other trypanosomatids. Work on the mechanism of methotrexate resistance in *Leishmania* has dramatically increased our understanding of folate and pterin metabolism in this organism. The metabolic and cellular functions of the reduced form of folates and pterins are beginning to be established and this work has led to several unexpected findings. Moreover, the currently ongoing sequencing efforts on trypanosomatid genomes are suggesting the presence of several gene products that are likely to require folates and pterins. A number of the properties of folate and pterin metabolism are unique suggesting that these pathways are valid and worthwhile targets for drug development. © 2002 Published by Elsevier Science Ltd. on behalf of Australian Society for Parasitology Inc.

Keywords: Biopterin; Cellular transport; Drug resistance; Folate metabolism; *Leishmania*; Methotrexate

1. Introduction

Leishmania is a protozoan parasite that is distributed worldwide, being endemic in 88 countries. The life cycle of *Leishmania* is relatively simple where they are found as motile flagellated promastigotes in the alimentary tract of the sand fly vector. Within the insect, the parasites can differentiate into the highly infectious metacyclic form. During a blood meal, the parasites are transmitted and engulfed by vertebrate macrophages, where they will then transform into the amastigote stage and divide within the acidified phagolysosomes (Burchmore and Barrett, 2001). Depending on the infecting species, *Leishmania* can give rise to a number of diseases including cutaneous, mucocutaneous and visceral infections. No effective vaccines are available against *Leishmania* infections as yet (Handman, 2001) and treatment relies on chemotherapy. The chemotherapeutic arsenal is limited and includes pentavalent antimonials as first-line drugs and amphotericin B and pentamidine as second-line drugs (Herwaldt, 1999; Murray, 2000).

Resistance to antimony is now observed in several parts of the world (Faraut-Gambarelli et al., 1997; Grogl et al., 1992; Jackson et al., 1990), most notably in the state of Bihar, India, where more than 50% of the patients are unresponsive to treatment, or relapse after conventional chemotherapy (Lira et al., 1999; Sundar et al., 2000). Some of the biochemical differences present in *Leishmania*, such as the ergosterol biosynthetic pathway, the exquisite requirements for purine salvage, and the high levels of ether lipids, have led to the use of ketoconazole, allopurinol and miltefosine as novel anti-leishmanial drugs. With the exception of miltefosine (Jha et al., 1995), these drugs are not useful against visceral *Leishmania*. Clearly, novel drug targets are urgently required for the treatment of *Leishmania* infections and a better understanding of novel biochemical pathways would be helpful in the development of new drugs for the treatment of *Leishmania* and other trypanosomatid parasites.

A biochemical pathway that has been exploited in the past in the treatment of infectious diseases is the folic acid biosynthetic pathway. Folic acid consists of three building blocks: a pterin, *p*-aminobenzoic acid (pABA) and glutamic

* Corresponding author. Tel.: +1-418-654-2705; fax: +1-418-654-2715.
E-mail address: marc.ouellette@crchul.ulaval.ca (M. Ouellette).

[1] Present address: Microcide Pharmaceuticals, Mountain View, CA, USA.

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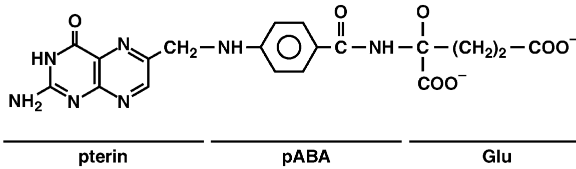

Fig. 1. Structure of folic acid. Folic acid is composed of three building blocks: a pterin moiety, *p*-aminobenzoic acid (pABA) and glutamic acid (Glu). In most organisms, folates are polyglutamylated.

acid (Fig. 1). Analogues of folic acid that can inhibit cellular growth are called antifolates. Trimethoprim is an antibiotic used in the treatment of a wide variety of bacterial diseases, and folic acid antagonists such as pyrimethamine have been useful in the treatment of infections due to apicomplexan parasites such as *Plasmodium spp.* and *Toxoplasma*. These drugs, however, are not effective in the treatment of infections due to trypanosomatid parasites such as *Leishmania* or against the aetiological agents of sleeping sickness, *Trypanosoma brucei*, and Chagas disease, *Trypanosoma cruzi*. Our understanding of folate and pterin metabolism and transport in trypanosomatids has stemmed largely from studies done on the mechanisms of resistance to the model antifolate drug methotrexate in *Leishmania*. These studies have unravelled new roles for pterins in cell physiology and parasite biology, and have pinpointed potential novel drug targets that could be exploited.

2. Biosynthesis of pterins and folates

Several microorganisms and higher eukaryotic cells have the ability to synthesise pterin derivatives de novo from GTP (Fig. 2), although in mammals reduced pterin in the form of tetrahydrobiopterin (BH_4) is formed. The first and rate-limiting step is catalysed by GTP cyclohydrolase I. This enzyme appears to be lacking in *Leishmania* and other trypanosomatid parasites but is present, for instance, in *Plasmodium falciparum* (Lee et al., 2001). The other two enzymes involved in BH_4 biosynthesis in higher eukaryotes are 6-pyruvoyl-tetrahydropterin synthase and sepiapterin reductase (Fig. 2). *Leishmania*, in contrast to humans, is a pterin auxotroph and has thus developed a sophisticated pterin salvage pathway that will be discussed below. Pterins are essential for the growth of several *Leishmania* species and other trypanosomatid parasites (Trager, 1969), and pterins including biopterin have been demonstrated to reduce the requirements for folates in *Crithidia fasciculata* (Kidder and Dutta, 1958).

Folate is a generic name for several structurally similar compounds that serve as co-factors in a variety of one-carbon transfer reactions (Fig. 3). The reduced form of folate can exist as various one-carbon derivatives including 5-methyl-, 10-formyl-, 5,10-methylene-, and 5,10-methenyltetrahydrofolate. The main known function of reduced folates in *Leishmania* is in the biosynthesis of thymidylate (Fig. 3). Folates are also implicated in metabolic pathways leading to methionine biosynthesis and purine biosynthesis, in the interconversion of serine and glycine, and in histidine catabolism (Fig. 2). *Leishmania* are purine auxotrophs (Hammond and Gutteridge, 1984), thus, folates are not important for purine biosynthesis. Most bacteria, fungi and several parasites such as *Plasmodium* are capable of synthesising folates de novo. Organisms that can synthesise folates de novo need to conjugate the pterin to pABA to form dihydropteorate. This is catalysed by the enzyme dihydropteorate synthase (Fig. 2). Dihydropteorate synthase is an excellent chemotherapeutic target against which sulfonamides are directed. Organisms incapable of folate synthesis, including mammals and *Leishmania*, rely on folates from the environment and have a need for specific transport systems and receptors to satisfy their folate requirements.

The enzyme dihydrofolate synthase catalyses the addition of glutamic acid to 7,8-dihydropteorate leading to the synthesis of 7,8-dihydrofolate. The enzyme dihydrofolate reductase reduces dihydrofolate to tetrahydrofolate (Fig. 2). This reduced folate is modified to 5,10-methylenetetrahydrofolate to serve as a carbon donor for the synthesis of thymidylate in a reaction catalysed by thymidylate synthase (Fig. 3). In *Leishmania*, as in other protozoa and several plants, dihydrofolate reductase and thymidylate synthase are fused

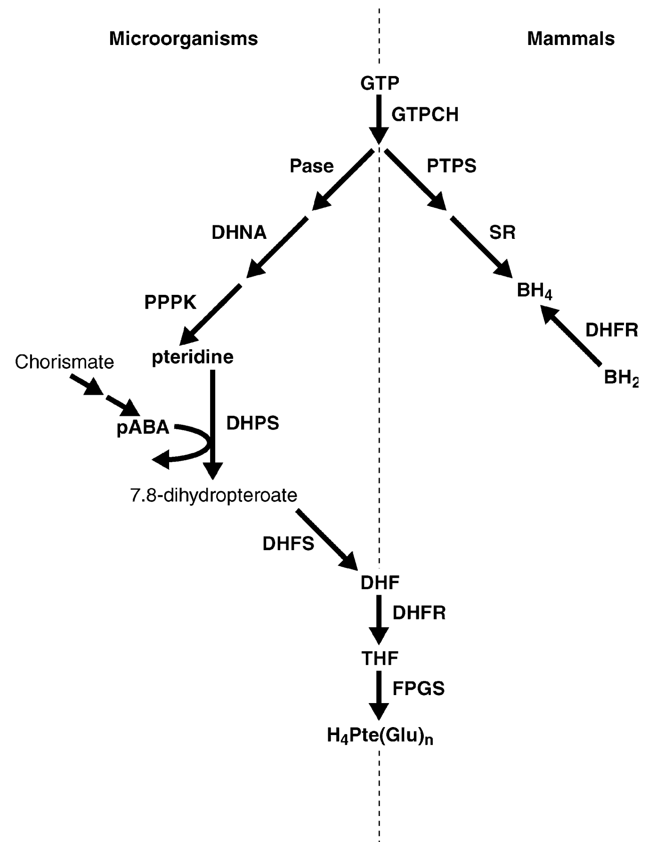

Fig. 2. De novo biosynthesis of pterins and folates. Both pterin and folates can be synthesised from GTP. Trypanosomatids cannot synthesise folic acid or biopterin and must obtain them from the environment.

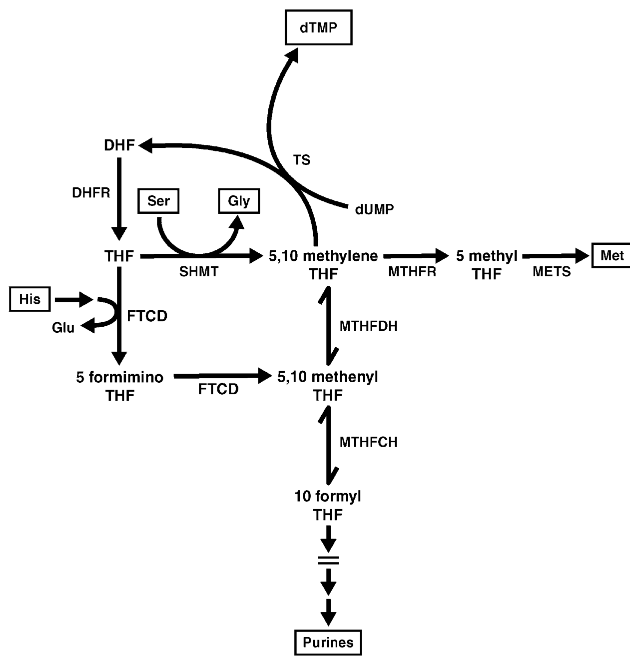

Fig. 3. Cellular functions of reduced folates. Folates in their reduced forms are involved in the biosynthesis of thymidine, the conversion of serine to glycine, methionine biosynthesis, histidine catabolism, and purine biosynthesis. The first three functions were shown to occur in *Leishmania* (or are likely to occur since the *Leishmania* genome contains the relevant genes), the histidine catabolic pathway has not been studied and purine biosynthesis does not occur in trypanosomatids. Abbreviations are listed in Table 1.

resulting in a bifunctional dihydrofolate reductase–thymidylate synthase protein (Beverley et al., 1986; Ferone and Roland, 1980; Grumont et al., 1986).

Intracellular folates in microorganisms and animal cells occur primarily as polyglutamates with four to six residues (Moran, 1999; Shane, 1989). The polyglutamylation is carried out by the enzyme folylpolyglutamate synthase (Fig. 2), an enzyme that shows some sequence similarity to dihydropteorate synthase. The physiological importance of folylpolyglutamates is now well established; they are more efficiently retained in the cell, are often better substrates or cofactors for some of the enzymes involved in folate metabolism, and finally, are better accumulated in mitochondria (Moran, 1999; Shane, 1989). The current state of knowledge and understanding of the synthesis and role of pterins and folates in *Leishmania* biology will be reviewed in the following sections.

3. Pterin metabolism in *Leishmania*

3.1. Pterin reductase PTR1

One of the first genomic loci amplified upon methotrexate selection in several *Leishmania* species corresponds to the H locus, a 40 kb region of chromosome 23 (Beverley et al., 1984; Chiquero et al., 1994; Hightower et al., 1988; Papadopoulou et al., 1993; Petrillo-Peixoto and Beverley, 1988; White et al., 1988). This locus is also amplified in *Leishmania* cells selected for resistance to a number of unrelated drugs including primaquine (Ellenberger and Beverley, 1989), arsenite (Detke et al., 1989; Ouellette et al., 1991) and antimony (Haimeur et al., 2000). Several resistance genes were found to be present at the H locus, and the metal (arsenite and antimony) resistance gene was identified as the ABC transporter P-glycoprotein related gene A (Ouellette et al., 1990). Recent work indicated that P-glycoprotein related gene A confers heavy metal resistance by sequestering metal–thiol conjugates within an intracellular organelle (Légaré et al., 2001). Amplification of the H locus following methotrexate selection served as a paradigm for the elucidation of several of the mechanisms of gene amplification in *Leishmania*. Indeed, repeated sequences present at the H locus were proposed (White et al., 1988), found (Ouellette et al., 1991) and proved (Grondin et al., 1996) to be important for the generation of extrachromosomal amplicons with either direct or inverted amplified sequences. Studies of the amplification of the H locus following methotrexate selection also indicated that linear amplicons could be precursors of circular amplicons (Grondin et al., 1998) and that the resistance gene and the genomic environment both influence the frequency of gene amplification (Kündig et al., 1999b).

Methotrexate is a potent inducer of the amplification of the H locus either as part of circular or linear amplicons. One of the first applications of gene transfection technology in *Leishmania* has been in revealing the methotrexate resistance gene present on the H locus. The gene, first named *LTDH* (Papadopoulou et al., 1992) and *HMTXR* (Callahan and Beverley, 1992) was found to code for a gene product belonging to the short-chain dehydrogenase reductase family. Members of the short-chain dehydrogenase reductase family are enzymes involved in several oxidation/reduction reactions (Jornvall et al., 1995). A number of roles were proposed for the *Leishmania* short-chain dehydrogenase reductase, but it was noteworthy that dihydropteridine reductase, the enzyme that recycles quinonoid dihydrobiopterin (Varughese et al., 1994), and sepiapterin reductase, the last enzymatic step in the mammalian de novo BH_4 biosynthesis pathway (Fig. 2), are also part of the short-chain dehydrogenase reductase family. Disruption of the *Leishmania* short-chain dehydrogenase reductase gene led to methotrexate hypersensitivity and to defects in pterin metabolism (Bello et al., 1994; Papadopoulou et al., 1994). Biochemical studies indicate that this enzyme, renamed PTR1, is a NADPH-dependent pterin reductase active as a tetramer (Nare et al., 1997a; Wang et al., 1997). In addition to reducing biopterin to BH_2 and BH_4, PTR1 is also capable of reducing folate to 7,8-dihydrofolate and tetrahydrofolate (Fig. 4). PTR1 was shown to contribute about 10% of the reduction of folates in wild-type cells while the remaining 90% was due to the activity of dihydrofolate reductase–thymidylate synthase (Nare et al., 1997a). Since PTR1 is significantly less inhibited by methotrexate than dihydrofolate reductase–thymidylate

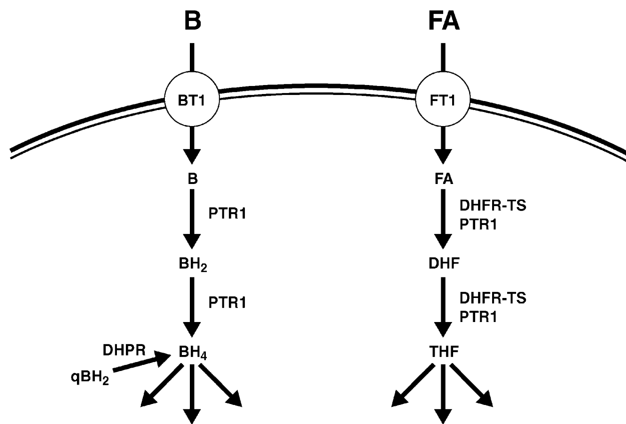

Fig. 4. Biopterin and folate transport and reduction in *Leishmania*. Leishmania has developed transport systems for both biopterin (B) and folic acid (FA) and the appropriate enzymes for their reduction to their bioactive form. The completely reduced forms of biopterin and folic acid, BH_4 and THF, are involved in a number of metabolic and cellular functions (see Figs. 3 and 5). Abbreviations are listed in Table 1.

synthase (Nare et al., 1997a), this activity allows it to act as a metabolic by-pass of dihydrofolate reductase–thymidylate synthase. This resistance mechanism is reminiscent of the alternative plasmid-encoded bacterial dihydrofolate reductase that confers resistance to the antifolate trimethoprim (Ouellette et al., 1998). The active site of PTR1 has been studied by site-directed mutagenesis (Chang et al., 1999; Leblanc et al., 1998) and more recently the crystal structure of PTR1 was solved (Gourley et al., 2001). Interestingly, the enzyme applies two distinct reduction mechanisms to substrates bound in one orientation. One mechanism is reminiscent of short-chain dehydrogenase reductase, while the second one is similar to the mechanism shown for dihydrofolate reductase (Gourley et al., 2001). Clearly, if antifolate chemotherapy is to be developed against *Leishmania*, it must target both dihydrofolate reductase and PTR1 activities.

Outside *Leishmania*, genes homologous to PTR1 have been observed only in the kinetoplastids *T. cruzi* (Robello et al., 1997) and in *T. brucei* (S. Detke, NCBI Accession number AF049903). Homologous transfection of the *T. cruzi PTR1* gene confers methotrexate resistance in the *T. cruzi* epimastigote (Robello et al., 1997). In *Leishmania*, PTR1 is expressed throughout the infectious cycle of the parasite (Cunningham and Beverley, 2001), whereas it is only observed in the epimastigote stage of the *T. cruzi* parasite and not in the amastigote or trypomastigote forms (Robello et al., 1997). The lack of PTR1 in the *T. cruzi* amastigote is intriguing and it is possible their ability to import host-reduced pterins has surpassed that of *Leishmania*.

3.2. The biopterin transporter BT1

Leishmania cells can transport biopterin (Beck and Ullman, 1991; Cunningham et al., 2001; Kündig et al., 1999a; Lemley et al., 1999) and the plasma membrane biopterin transporter BT1 was isolated by functional cloning using two different selective strategies. In one case, resistance to methotrexate was the selective marker, while in the other case complementation of a methotrexate-resistant folate transport mutant was used (Kündig et al., 1999a; Moore, J., Beverley, S., 1996. Woods hole parasitology meeting, abstract 107.). Interestingly, the biopterin transporter called BT1 (Lemley et al., 1999) corresponds to ORFG (Myler et al., 1994), a gene product that was in search of a function. *ORFG* is part of the LD1 locus that is frequently amplified in several species of *Leishmania* isolated either from the laboratory or from the field (Segovia and Ortiz, 1997; Tripp et al., 1991). BT1, a transmembrane protein containing 12 putative membrane-spanning domains, has been localised to the plasma membrane using a BT1-green fluorescent protein fusion (Kündig et al., 1999a). Biochemical analysis of BT1 transfectants strongly suggests that BT1 is both a high-affinity biopterin transporter and a low-affinity folate transporter (Kündig et al., 1999a; Lemley et al., 1999). BT1 is the only non-conjugated pterin transporter presently known. The disruption of the *BT1* gene has been achieved in *Leishmania tarentolae* (Kündig et al., 1999a), in *Leishmania donovani* (Lemley et al., 1999; Papadopoulou et al., 2002), and in *Leishmania major* (Cunningham et al., 2001). In *BT1* mutant strains, biopterin uptake is completely abolished, further suggesting that BT1 is likely the main biopterin transporter of *Leishmania* cells. Pterin supplementation was nonetheless essential for growth of the BT1 mutant. It is possible that in the absence of BT1, biopterin may enter the cell by diffusion (Lemley et al., 1999; Papadopoulou et al., 2002).

Overexpression of BT1 confers methotrexate resistance, possibly because in addition to the transport of biopterin, BT1 permits the selective transport of folate but not of methotrexate (Kündig et al., 1999a). Thus, sufficient folates are transported into the cell through BT1 to allow for cellular growth. In this scenario, BT1 would be able to confer resistance only in relatively folate-rich medium. The sensitivity of *Leishmania* cells to methotrexate is highly dependent on the folate concentration of the medium (Kaur et al., 1988; Papadopoulou and Ouellette, 1993; Peixoto and Beverley, 1987). In all *L. tarentolae* methotrexate-resistant mutants in which there was a marked reduction in the activity of the folate/methotrexate transporter, BT1 is overexpressed following gene rearrangement, possibly allowing sufficient folates to enter these mutants (Kündig et al., 1999a). It is possible that *BT1* gene amplification will be observed in several *Leishmania* species selected for methotrexate resistance in which folate uptake is reduced. For example, a DNA region containing *BT1* was amplified in *L. major* selected for methotrexate resistance (Beverley and Coburn, 1990).

Proteins similar to BT1 are found exclusively in trypanosomatid parasites. Ongoing genome projects have revealed putative homologues in *T. brucei* and *T. cruzi*. BT1 shares considerable homology with ESAG10 of *T. brucei*, a protein with unknown function encoded by an expression site-asso-

ciated gene (Gottesdiener, 1994). It is possible that ESAG10 is a pteridine transporter (either conjugated or not) although our preliminary attempts to prove this were unsuccessful (Kündig et al. unpublished). The *Leishmania BT1* gene product is active in both main life stages, although the kinetics of biopterin uptake are more complex in the *Leishmania mexicana* amastigote than in the promastigote (Cunningham and Beverley, 2001). The stage-specific expression of *BT1* homologs in other kinetoplastid parasites is unknown.

3.3. Regeneration of reduced pterins

Upon the conversion of phenylalanine to tyrosine, BH_4 is converted to a quinonoid dihydrobiopterin, (qBH_2) (Thony et al., 2000). This quinonoid substrate is reduced back to BH_4 by dihydropterin reductase, an enzyme falling in the same branch of the short-chain dehydrogenase reductase family as PTR1 (Varughese et al., 1994). However, PTR1 has no activity towards qBH_2 (Wang et al., 1997). Dihydropteridine reductase activity has been described in *C. fasciculata* (Hirayama et al., 1980) and in *Leishmania* (Bello et al., 1994) but the corresponding genes have not yet been isolated. Since *Leishmania* can recycle quinonoid pterin, it is likely that this molecule is produced following BH_4-dependent enzymatic reactions; however, these pathways are still unknown in *Leishmania*.

3.4. Pterins and putative de novo folate biosynthesis in Leishmania

The exact functions of pterins in *Leishmania* still need to be established. Several lines of evidence suggest their involvement in the biosynthesis of folates. Indeed, although *Leishmania* cells rely predominantly on the uptake of extracellular folate for their folate requirements, *Leishmania* cells without measurable folate transport grow perfectly well under standard laboratory conditions (Kaur et al., 1988; Papadopoulou et al., 1993). It was thus proposed that these mutants obtained folates by other routes of entry or by increased de novo synthesis. An important enzyme in de novo synthesis is DHPS (Fig. 2) but *Leishmania* appears to lack this activity and, indeed, none of the sulfonamides tested has shown activity against *Leishmania* (Kaur et al., 1988; Peixoto and Beverley, 1987). If *Leishmania* has the ability to conjugate the pterin to pABA, it must do so with an enzyme that bares little similarity to conventional DHPS. Nonetheless, several *Leishmania* species can grow in completely defined folate-free medium provided that a pterin source is available. Indeed biopterin and a variety of other pterins can eliminate the folate requirements in *L. donovani*, *L. major*, *L. mexicana* and *L. tarentolae* (Beck and Ullman, 1990; Nare et al., 1997b; Papadopoulou et al., 1994; Peixoto and Beverley, 1987). The ability of pterins to support growth correlates well with their activity as PTR1 substrates. At least two interpretations of the ability of biopterin to support the growth of *Leishmania* species in folate-deficient medium have been suggested. One possibility is that *Leishmania* can synthesise folates de novo if a pterin source is available. Alternatively, folate-deficient medium is not totally free of folates, and trace contaminants could support growth. Biopterin or other unconjugated pteridines would dramatically reduce the requirement for folates, a sparing effect that was first described in *C. fasciculata* almost 25 years ago (Kidder and Dutta, 1958).

The demonstration that *L. donovani* could convert [^{3}H]biopterin into 5-methyltetrahydrofolate and 10-formyltetrahydrofolate (Beck and Ullman, 1991) was obviously a strong argument for the ability of *L. donovani* to synthesise folates from pterins by an unconventional route. However, at least in *L. major*, radioactive pABA was not incorporated into folates (Kovacs et al., 1989). This argues against de novo synthesis, but one could propose that folate synthesis is clearly not proceeding through a DHPS pathway. The ability of *Leishmania* cells to convert pterins into folates remains to be confirmed. It is thus possible that *Leishmania* cells are both pterin and folate auxotrophs and that the growth of *Leishmania* cells in defined medium supplemented with pterins is due to a folate sparing effect. *Leishmania* cells can grow in medium in which folate concentration is less than 0.01 nM when biopterin is present (Peixoto and Beverley, 1987), making this effect both pronounced and intriguing.

How does this explain the ability of methotrexate-resistant mutants to grow in absence of measurable folate uptake? This paradox was partly solved by studying *L. tarentolae* methotrexate-resistant mutants, which exhibited no folate uptake (Kündig et al., 1999a). BT1 was overexpressed in these mutants, and since BT1 is a low-affinity folate transporter, enough folate can be transported by BT1 when high concentrations of folates are present. It remains to be seen, however, whether a deficit in folate transport is always compensated for by increased expression of BT1. This does not seem to be the case for at least one *L. donovani* mutant, where biopterin uptake was unchanged while there was no measurable folate/methotrexate uptake (Beck and Ullman, 1991).

3.5. Pterin as an essential growth factor

The essential role of pterin in cell physiology was first appreciated by the demonstration that BH_4 is an essential growth factor for *C. fasciculata* (Kidder and Dutta, 1958). More recently, BH_4 has been shown to have proliferative activity in a number of mammalian cell lines (reviewed in Thony et al., 2000). The role of pterins in *Leishmania* physiology is likely to be as important. Indeed, the *PTR1* gene, as part of the H locus, has been found amplified in several unselected stocks of different *Leishmania* species (Hightower et al., 1988; Petrillo-Peixoto and Beverley, 1988; White et al., 1988). Similarly, the *BT1* gene, as part of the LD1/C1 extrachromosomal element, is frequently

amplified in unselected field strains (Segovia and Ortiz, 1997; Tripp et al., 1991). It is therefore possible that under specific circumstances within either the insect vector or the mammalian host, biopterin (or a related pterin) becomes limiting, and the overexpression of genes involved in pterin metabolism or transport provides a marked growth advantage. The growth in culture medium relatively poor in pterins of several strains of *L. major* and *L. donovani* freshly isolated from animals required the addition of biopterin. However, after numerous passages in this medium the cells adapted and biopterin supplementation was no longer necessary. In all of these adapted cell lines, either PTR1 or BT1 was overexpressed (Roy et al., 2001), suggesting that modulation of the expression of these genes can provide a marked growth advantage under pterin-limited conditions. The exact molecular mechanism by which pterins exert their growth promoting activity still needs to be established.

3.6. Putative role of pterins in Leishmania physiology and metabolism

Although pterins are unlikely to be important for folate biosynthesis in *Leishmania*, they are nevertheless powerful growth potentiators. A number of functions have been associated with BH_4 in mammalian cells (Fig. 5), and it remains to be seen whether similar functions will apply in *Leishmania* and related kinetoplastids.

3.6.1. BH_4-dependent amino acid hydroxylases

One of the best studied functions of BH_4 consists of its role as a natural cofactor for a small family of aromatic amino acid monooxygenases including phenylalanine hydroxylase, tyrosine hydroxylase, and tryptophan hydroxylase (Fitzpatrick, 1999). Phenylalanine hydroxylase catalyses the formation of tyrosine (Fig. 5) and mutation of this enzyme is a frequent cause of phenylketonuria. Tyrosine hydroxylase catalyses the first step in the biosynthesis of the catecholamine neurotransmitters, while tryptophan hydroxylase catalyses the first step in the biosynthesis of serotonin and melatonin (reviewed in Thony et al., 2000). The structures of eukaryotic aromatic amino acid hydroxylases are similar, consisting of a regulatory domain at the N-terminus, a central catalytic domain of ~300 amino acids, and a tetramerisation domain at the C-terminus. The bacterial hydroxylases contain only the catalytic domain (Fitzpatrick, 1999). Survey of the ongoing *Leishmania* genome project has revealed a putative homologue of the monooxygenases (Table 1). The full length gene was cloned and sequenced and the *Leishmania* enzyme carries the three domains of the eukaryotic enzymes (El Fadili et al., unpublished). The amino acid specificity, cofactor requirements, and functional role of this enzyme must be tackled experimentally, but sequence similarity (>50% identity with the human phenylalanine hydroxylase enzyme) suggests that this would be a *Leishmania* BH_4-requiring enzyme. It has been suggested that trypanosomatids lack phenylalanine hydroxylase activity and fail to convert phenylalanine to tyrosine (Kidder and Dewey, 1963). Moreover, no role for catecholamines and other neurotransmitters is known for *Leishmania*. The determination of the role of the *Leishmania* monooxygenase will thus be of great interest.

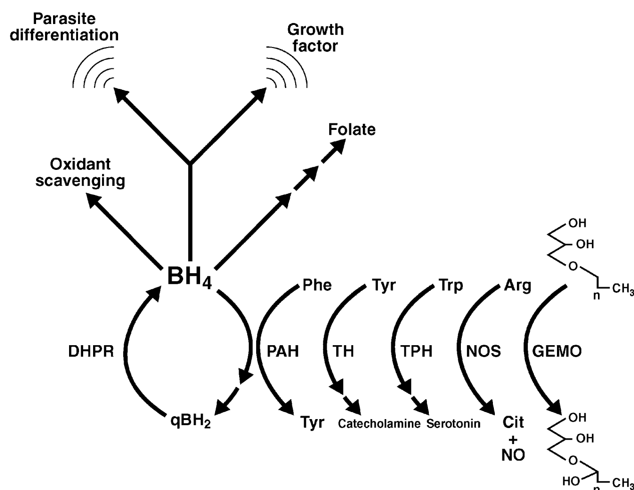

Fig. 5. Cellular functions of reduced pterins. Reduced pterins are involved in a number of metabolic and cellular functions. Some of these have been well studied, such as the hydroxylation of aromatic amino acids, while others highly relevant to trypanosomatids, such as growth promotion and parasite differentiation, have been conclusively demonstrated although are not yet well understood. Abbreviations are listed in Table 1.

3.6.2. Nitric oxide synthase

More recently, the results of a considerable amount of work clearly demonstrated that BH_4 is an essential cofactor of the three forms (inducible, neural and endothelial) of nitric oxide synthase in mammalian cells (reviewed in Marletta et al., 1998). Nitric oxide synthase catalyses the oxidation of arginine to citrulline and nitric oxide. Nitric oxide is a molecule with antimicrobial properties and plays a critical role in signal transduction pathways of the cardiovascular and nervous systems and in the immune response (Marletta et al., 1998). Nitric oxide synthase is found in higher eukaryotes, and although no gene sequence with similarities to eukaryotic nitric oxide synthases have yet been found in the 50% of the Leishmania genome sequenced to date, there is one report indicating that Leishmania may have nitric oxide synthase activity (Basu et al., 1997). The first non-animal nitric oxide synthase was described recently in *Physarum* (Golderer et al., 2001) and thus it may exist in other lower eukaryotes such as Leishmania.

3.6.3. Reactivity to oxidants

Strong scavenging activities of BH_4 for superoxide anion radicals have been described (Kojima et al., 1995; Nakamura et al., 2001). Nitric oxide toxicity involves H_2O_2 production, and scavenging of the latter by BH_4 may explain the prevention of cell death induced by nitric oxide donor (Thony et al., 2000). *L. major PTR1* null mutants are more sensitive while PTR1 overexpressers appear to have increased resistance to a

Table 1
Enzymes involved in pterin and folate metabolic pathways

Gene	Name	EC number	GenBank accession number(s) for *Leishmania* genes
BT1	Biopterin transporter		AAF77206, CAB95513
DHFR	Dihydrofolate reductase	1.5.1.3	AAC28092, AAK71186, AAB49898, AAA91362 , AAA91362, CAA36020, AAA29232, AAA30318
DHFS	Dihydrofolate synthase	6.3.2.12	CAC02007[a]
DHNA	Dihydroneopterin aldolase	4.1.2.25	Not likely to exist
DHPR	Dihydropteridine reductase	1.6.99.7	Not likely to exist
DHPS	Dihydropteorate synthase	2.5.1.15	Not likely to exist
FPGS	Folylpolyglutamate synthetase	6.3.2.17	AF284554
FT1	Folate transporter 1		AF08449
FTCD	Formiminotransferase cyclodeaminase	2.1.2.5 and 4.3.1.4	
GEMO	Glyceryl-ether mono oxygenase	1.14.16.5	
GTPCH	GTP cyclohydrolase I	3.5.4.16	Not likely to exist
METS	Methionine synthase	2.1.2.13	AQ848249[a]
MTHFR	Methylene tetrahydrofolate reductase	1.5.1.20	CAB97843[a]
MTHFDH	Methylene tetrahydrofolate dehydrogenase	1.5.1.5	
MTHFCH	Methenyl tetrahydrofolate cyclohydrolase	3.5.4.9	
NOS	Nitric oxide synthase	1.14.13.39	Not likely to exist
PAH	Phenylalanine hydroxylase	1.14.16.1	CAC00881[a]
PPPK	Hydromethyl-dihydropterin pyrophosphokinase	2.7.6.3	Not likely to exist
PTPS	6-Pyruvoyl-tetrahydropterin synthase	4.6.1.10	Not likely to exist
PTR1	Pterin reductase	1.1.1.253	Q01782, P4255, AAC38850, AF049903
SHMT	Serine hydroxymethyl transferase	2.1.2.1	CAB94023
SR	Sepiapterin reductase	1.1.1.153	
TH	Tyrosine hydroxylase	1.14.16.2	
TPH	Tryptophan hydroxylase	1.14.16.4	
TS	Thymidylate synthase	2.1.1.45	Fused to DHFR

[a] Partial gene sequence.

variety of reactive oxygen intermediates (Nare et al., 1997b). Primaquine induces *PTR1* gene amplification (Ellenberger and Beverley, 1989) and although PTR1 overexpressers are not more resistant to primaquine, *PTR1* null mutants are more sensitive (Bello et al., 1994). Since primaquine can induce oxidative stress (Vasquez-Vivar and Augusto, 1994), the increased sensitivity of a *PTR1* null mutant to primaquine may be due to the reduced ability of the mutant to deal with oxidants (Nare et al., 1997b). Thus, it is likely that PTR1-dependent reduced pterins are useful (in complement with other molecules e.g. trypanothione, superoxide dismutase) in dealing with oxidative stress in *Leishmania* and perhaps in other trypanosomatids.

3.6.4. Glyceryl–ether monooxygenase

Cleavage of ether-linked lipids by the enzyme glyceryl–ether monooxygenase requires a reduced pterin and oxygen (Kaufman et al., 1990). *Leishmania* surface lipophosphoglycan contains esterified fatty acids (Turco and Descoteaux, 1992). An ether-linked lipid cleavage activity was demonstrated in *Leishmania*, but was shown to use NADPH as a co-factor rather than BH_4 (Ma et al., 1997). Thus, BH_4 may not have an important role in the cleavage of ether-linked lipids in *Leishmania*.

3.6.5. Molybdopterin-containing enzymes

Molybdenum-containing enzymes are found in a wide range of organisms and catalyse the transfer of an oxygen atom in reactions involved in the nitrogen, sulfur and carbon cycles (Kisker et al., 1998). Molybdenum is coordinated to the sulfur atom of molybdopterin, a substituted pterin derivative, which is then incorporated in the enzyme. The roles of molybdopterin-containing enzymes have not yet been studied in *Leishmania*, but the ongoing sequencing of the genome has revealed at least one open reading frame (ORF) (AL161416) predicted to be the molybdopterin synthase sulphurylase, which shares 35% identity with several other molybdopterin synthase sulphurylase homologues. Molybdopterin synthase sulphurylase is involved in the synthesis of the molybdopterin co-factor. This molybdopterin is usually synthesised from guanosine (Irby and Adair, 1994; Wuebbens and Rajagopalan, 1995) but pterins, as shown in the yeast *Pichia canadensis*, can also be used for molybdopterin biosynthesis (Irby and Adair, 1994).

3.6.6. Regulation of parasite differentiation

Due to the central role of pterins in *Leishmania* biology, intuitively, one would expect that *BT1* and *PTR1* null mutants would be attenuated and would give rise to reduced infections in animal models. This was indeed the case for an

L. donovani BT1 null mutant (Papadopoulou et al., 2002) but surprisingly, an *L. major PTR1* null mutant was more infective in the mouse model (Cunningham et al., 2001). This increased virulence appears to be due to regulation of metacyclogenesis by BH_4, as both *BT1* and *PTR1* null mutants of *L. major* displayed both lower BH_4 levels and a specific increase in the proportion of metacyclic parasites (Cunningham et al., 2001). We have made similar observations while studying *L. donovani BT1* null mutants (Roy et al., unpublished data). The ability to transport pterins, in contrast to their reduction, apparently needs to remain intact for intracellular survival. The mechanism by which decreased levels of BH_4 increase metacyclogenesis is unknown. One possibility is that it is a cofactor for an enzyme involved in controlling differentiation; alternatively, it could be involved in a novel signaling pathway. Intriguingly, folates or pterins have been proposed to regulate developmental timing in *Dictyostelium* (Hadwiger et al., 1994; Salger and Wetterauer, 2000).

4. Folate metabolism in *Leishmania*

Although *Leishmania* is probably incapable of carrying out de novo synthesis of folates, they are nonetheless essential cofactors for several enzymes (Fig. 3). Early studies have suggested that *Leishmania* includes a number of folate-metabolizing enzymes including serine hydroxymethyltransferase, dihydrofolate reductase, thymidylate synthase, and methylenetetrahydrofolate reductase (Avila and Nosei, 1983; Nosei and Avila, 1985; Scott et al., 1987). When *Leishmania* cells are incubated with [^{3}H]folic acid, it is metabolised and 10-formyltetrahydrofolate and 5-methyltetrahydrofolate are the only intermediates that can be detected (Beck and Ullman, 1991; Scott et al., 1987).

4.1. Dihydrofolate reductase–thymidylate synthase

Amplification of the *DHFR-TS* gene in *Leishmania* (Coderre et al., 1983) was the first indication that drug resistance in a protozoan could be mediated by gene amplification. Overexpression of dihydrofolate reductase–thymidylate synthase leads to methotrexate resistance, although at significantly lower levels than seen with PTR1 overexpression (Kündig et al., 1999b). The sequence of the *Leishmania* gene has confirmed that dihydrofolate reductase is fused to thymidylate synthase (Beverley et al., 1986; Grumont et al., 1986), and the bifunctional enzyme catalyses consecutive reactions in the de novo synthesis of dTMP (Fig. 3). *DHFR-TS* null mutants are thymidine auxotrophs (Cruz et al., 1991), confirming that the main role of the protein is in thymidine biosynthesis. Interestingly, although it is relatively easy to generate *DHFR-TS* null mutants of *L. major* laboratory strains, it appears that the same is not possible for highly virulent strains (Cruz et al., 1993), suggesting that folates may have important roles other than in thymidine biosynthesis. *L. major DHFR-TS* null strains are attenuated and can protect mice against challenge with wild-type *Leishmania* (Titus et al., 1995). Amplification of *DHFR-TS* is frequently observed in *L. major* strains selected for methotrexate resistance (Ellenberger and Beverley, 1987b) but not in other species. For instance, *DHFR-TS* has never been amplified in *L. tarentolae* except when methotrexate selection is initiated from a *PTR1* null mutant. This could be due to a combination of factors, the locus where possibly specialised elements such as long repeated sequences increase the likelihood of locus amplification and the resistance gene itself (Kündig et al., 1999b).

The bifunctional nature of dihydrofolate reductase–thymidylate synthase has led to numerous studies on substrate channelling, whereby a metabolic intermediate is directly transferred from one enzyme active site to the next without being released into solution (Meek et al., 1985). The structure of dihydrofolate reductase–thymidylate synthase is known (Knighton et al., 1994) and has permitted the realisation that 7,8-dihydrofolate moves between the active sites of dihydrofolate reductase and thymidylate synthase across the surface of the protein. This channeling effect has also been confirmed by detailed enzyme kinetic analysis (Liang and Anderson, 1998).

4.2. Polyglutamylation of folates

In most organisms that have been analysed, folates exist mainly as polyglutamate derivatives linked to the γ-carboxyl group of the side chain of folates (Moran, 1999; Shane, 1989). The physiological importance of folylpolyglutamates is now well established; they increase the cellular retention of folates, they are often better substrates or cofactors for some of the enzymes involved in folate metabolism, and finally folylpolyglutamates are better accumulated in mitochondria where they are necessary for glycine biosynthesis (Moran, 1999; Shane, 1989). Polyglutamylation is carried out by the enzyme folylpolyglutamate synthetase and the levels of polyglutamylation of folates and antifolates are important factors in chemotherapy (Moran, 1999). In one study, it was found that folates in *Leishmania* are polyglutamylated, with tetra- and penta-glutamates being the predominant forms (Santi et al., 1987) but in contrast to mammalian cells, methotrexate was not polyglutamylated in *L. major* (Ellenberger et al., 1989).

Decreased polyglutamylation of methotrexate in mammalian cells leads to efflux of methotrexate and thus decreased retention. Altered transcriptional regulation of folylpolyglutamate synthase is correlated with acquired antifolate resistance (Longo et al., 1997). Methotrexate polyglutamates are transported into lysosomes where they are hydrolysed to the monoglutamate form by γ-glutamyl hydrolase. Enhanced activity of this enzyme can also lead to the acquisition of antifolate resistance (Rhee et al., 1993). We have recently observed that methotrexate is polyglutamylated in wild-type *L. tarentolae*, and that shorter gluta-

mate chains of methotrexate are found in methotrexate-resistant mutants while glutamate chains of folates remain at wild-type length (El Fadili et al., unpublished data). *Leishmania major* can hydrolyse methotrexate to *N*-10-methyl-4-deoxy-4-aminopteroate, a metabolite that is a less potent inhibitor of dihydrofolate reductase than methotrexate (Ellenberger et al., 1989) and this hydrolase activity has also been reported in *L. donovani* (Kaur et al., 1988) and in *C. fasciculata* (Oe et al., 1983). It could theoretically provide methotrexate resistance by hydrolysing it but this has not yet been demonstrated.

4.3. Folate transporters

In cells that depend on folates from the environment, mutation in the folate transporter will lead to decreased antifolate transport and hence to increased resistance (Gorlick et al., 1997). *Leishmania* have a common folate/methotrexate transporter and mutations in the gene for this transporter lead to methotrexate resistance (Dewes et al., 1986; Ellenberger and Beverley, 1987b; Kaur et al., 1988; Papadopoulou et al., 1993). Two mutant transport phenotypes have been observed in *Leishmania*; one that decreases uptake two- to five-fold, and one that decreases uptake more than 50-fold (Ellenberger and Beverley, 1987b; Kaur et al., 1988; Papadopoulou et al., 1993). Although these cells can now resist antifolates, they must compensate for the lack of folate uptake. *Leishmania tarentolae* methotrexate resistant mutants with profound defects in folate/methotrexate transport did overexpress BT1 through which some folate can be transported (Kündig et al., 1999a).

Southern hybridization with a *BT1* probe results in a number of hybridising bands indicating that BT1 is part of a multigene family with more than six new members which are part of a locus (Cunningham and Beverley, 2001; Kündig et al., 1999a). A deletion of part of the locus was identified in the methotrexate-resistant *L. tarentolae* mutants with severe folate/methotrexate uptake deficiencies. Transfection of one of the genes that is part of this locus into *Leishmania* cells confirmed that it corresponded to a folate transporter (Richard et al., unpublished data). It remains to be seen whether inactivation of this gene in the wild-type cell will lead to loss of uptake and methotrexate resistance.

The expression profiles of the members of the folate transporter gene family including FT1 were studied and found to be complex, involving differential expression of genes according to the life cycle of the parasite (Cunningham and Beverley, 2001). The capacities and affinities of the various transporters in this gene family for folate and pterin transport have not yet been investigated. It is possible that some of these transporters are specific for other pterins or are located either in the plasma membrane or in membranes of intracellular organelles. This gene family also appears to be present in *T. brucei*, as suggested by database mining (www.sanger.ac.uk/Projects/T_brucei).

Efflux of the antifolate methotrexate has been described in bacteria (Kopytek et al., 2000), in *Candida albicans* (Ben-Yaacov et al., 1994), and in mammalian cells. The reduced folate carrier of mammalian cells mediates bi-directional transport of methotrexate and steady-state accumulation of the drug is dependent on the combined rates of influx and efflux (Zhao et al., 1997). Other routes of methotrexate efflux have also been described in mammalian cells (Saxena and Henderson, 1996) and recently some members of the MRP family of ABC transporters were shown to produce methotrexate resistance (Hooijberg et al., 1999). Efflux of methotrexate has been observed in wild-type *L. major* cells (Ellenberger and Beverley, 1987a) and this could be further studied using everted membrane vesicles. Everted vesicles have been useful for the study of other drug resistance efflux pumps in *Leishmania* (Dey et al., 1996). In a methotrexate-resistant *L. tropica* mutant, an ABC transporter (PGPE) of the MRP family was found to be overexpressed (Gamarro et al., 1994). Although transfection of *PGPE* failed to produce methotrexate resistance, the role of PGPE in methotrexate resistance merits further investigation.

4.4. Serine hydroxymethyltransferase

Serine hydroxymethyltransferase catalyses the interconversion of serine and glycine, with tetrahydrofolate serving as the one carbon acceptor (Fig. 3). This activity has been described in a number of trypanosomatid parasites (Capelluto et al., 1999, 2000; Nosei and Avila, 1985; Scott et al., 1987) and the *Leishmania* genome project clearly indicates that the corresponding gene is present in *Leishmania* (Table 1) and shares more than 50% identity with yeast and mammalian serine hydroxymethyltransferases. The serine hydroxymethyltransferase enzyme was characterised in detail in *C. fasciculata* and *T. cruzi*. While three isoforms of serine hydroxymethyltransferase were observed in *C. fasciculata* (Capelluto et al., 1999), a single form of serine hydroxymethyltransferase was detected in epimastigotes of *T. cruzi* (Capelluto et al., 2000). *Crithidia fasciculata* subcellular fractionation data suggest that the isoforms of serine hydroxymethyltransferase are located in the cytosol, the mitochondria and possibly the glycosome (Capelluto et al., 1999). This emphasises our ignorance concerning the site(s) of folate and pterin metabolism in trypanosomatids. In eukaryotes, folate-mediated one-carbon metabolism is compartmentalised between the cytosol and mitochondria (reviewed in Appling, 1991), and it is important to determine where these metabolic reactions are taking place in trypanosomatids.

4.5. Methionine biosynthesis

The folate cofactor 5,10-methylenetetrahydrofolate produced via serine hydroxymethyltransferase is a substrate for methylenetetrahydrofolate reductase leading to 5-methyltetrahydrofolate (Fig. 3). Methionine synthase catalyses the transfer of a carbon group from this intermediate to produce methionine (Fig. 3). Although little is known about the methionine biosynthetic pathway in trypanosomatids,

the *Leishmania* genome project has revealed that *Leishmania* have both methylenetetrahydrofolate reductase and methionine synthase homologues (Table 1). Thus, it is probable that reduced folates are important for methionine biosynthesis in *Leishmania*.

4.6. Other functions of folate in Leishmania

As trypanosomatids are purine auxotrophs (Hammond and Gutteridge, 1984), reduced folates are unlikely to be important in this pathway. In several organisms, reduced folates are involved in histidine catabolism to glutamic acid (Fig. 3), which is mediated by the bifunctional enzyme formiminotransferase cyclodeaminase where the formiminotransferase activity transfers a formimino group to tetrahydrofolate while the cyclodeaminase converts 5-formiminotetrahydrofolate to 5,10-methenyltetrahydrofolate (Fig. 3). Interestingly, formiminotransferase cyclodeaminase seems to be involved in Golgi dynamics (Gao and Sztul, 2001). It is not known whether this pathway is operative in trypanosomatids. It is likely that folates have numerous roles in *Leishmania* biology that future work should help to unravel.

5. From metabolic pathways to chemotherapeutic intervention and prevention

Although antifolates are useful drugs in the treatment of a variety of infectious diseases, they have not yet been useful in the treatment of *Leishmania* or of other trypanosomatid infections. The widely used antimicrobial antifolates pyrimethamine and trimethoprim are poor inhibitors of the *Leishmania* dihydrofolate reductase (Sirawaraporn et al., 1988). Since the structure of the *Leishmania DHFR-TS* gene is known (Knighton et al., 1994), it should be possible to rationally design inhibitors. This possibility was recently put to proof when the dihydrofolate reductase domains of *Leishmania*, *T. brucei*, and *T. cruzi* were modeled (Zuccotto et al., 1998) and variations from the host enzymes provided a basis for selective drug design. A series of compounds were then synthesised, with some showing specificity for the parasite enzymes. A number of these inhibitors showed promising activity against an animal model of African trypanosomiasis (Chowdhury et al., 1999, 2001). Further work should permit the synthesis of an anti-dihydrofolate reductase with enhanced activity. Similarly, trypanosomatids contain several of the enzymes in the folate and pterin metabolic pathways (Figs. 2–5) that are targets for cancer chemotherapy. If structural or functional differences between the parasite and human enzymes are found, some of these should be attractive targets for chemotherapy.

The ability of PTR1 to circumvent the need for dihydrofolate reductase (Fig. 4) suggests that both of these enzymes would have to be targeted simultaneously in a successful antileishmanial drug strategy. This realisation has led to an effort to test a large collection of pteridine analogs for inhibitors of PTR1 (Hardy et al., 1997). Promising lead compounds have been isolated through these studies, some with good in vitro anti-*Leishmania* activity. This work suggested, however, that *Leishmania* cells may contain multiple targets for some of these pteridine analogs (Hardy et al., 1997). A combination of anti-dihydrofolate reductase and anti-PTR1 inhibitors, or an inhibitor having good activities against both enzymes, is a rational way of targeting the folate pathway as these activities are expressed in the amastigote stage (Cunningham and Beverley, 2001). If mutations in dihydrofolate reductase were to arise, a rare event in *Leishmania* (Arrebola et al., 1994), and subsequently abolish inhibition by antifolates, inhibiting PTR1 alone may reverse the desired effect since this could lead to hypervirulent parasites (Cunningham et al., 2001). The pterin and folate transporters (Fig. 4), which have no other homologues outside the trypanosomatid parasites, could provide a new class of target for inhibitory compounds.

No vaccines are available against trypanosomatid parasites. Recently, the BT1 protein was shown to provide partial protection against an infectious challenge of *L. donovani* in mice (Dole et al., 2000), and a *L. donovani BT1* null mutant showed a much reduced capacity of inducing infection in the same mouse model (Papadopoulou et al., 2002). *L. major* strains deficient in dihydrofolate reductase–thymidylate synthase are attenuated and give rise to protection against an infectious challenge in a mouse model (Titus et al., 1995). However, a *DHFR-TS* knock-out of *Leishmania chagasi* did not protect against subsequent *L. chagasi* challenge (Streit et al., 2001). This was proposed to be due to either the inability of the *L. chagasi DHFR-TS* null mutant to establish a subclinical infection or the loss of critical antigens following the genetic manipulations (Streit et al., 2001).

Although pterins have long been recognised as essential growth factors, we are still far from a complete understanding of their roles. Inactivation of PTR1 has led to the appreciation of an unexpected role for pterins in parasite differentiation (Cunningham et al., 2001), although other factors are involved in differentiation and pterins and folates are likely playing other roles. The ongoing parasite genome projects have pinpointed ORFs that are likely to be important in folate and pterin metabolism (Table 1) and the currently unsequenced half of the *Leishmania* genome will certainly reveal additional interesting genes. It is likely that proteomic approaches and DNA microarrays will be useful for determining cellular function of pterins and folates on a global scale and to further increase our understanding of folate and pterin metabolism in *Leishmania* and possibly in other organisms.

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