New approaches to *Leishmania* chemotherapy: pteridine reductase 1 (PTR1) as a target and modulator of antifolate sensitivity

B. NARE[1], J. LUBA[2], L. W. HARDY[2] *and* S. BEVERLEY[1]*

[1] *Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, 240 Longwood Avenue, Boston, MA 02115 USA*
[2] *Department of Pharmacology & Molecular Toxicology and Program in Molecular Medicine, University of Massachusetts Medical School, 373 Plantation St., Worcester MA 01605 USA*

SUMMARY

Leishmania and other trypanosomatid protozoa require reduced pteridines (pterins and folates) for growth, suggesting that inhibition of these pathways could be targeted for effective chemotherapy. This goal has not yet been realized, indicating that pteridine metabolism may be unusual in this lower eukaryote. We have investigated this possibility using both wild type and laboratory-selected antifolate-resistant strains, and with defined genetic knockouts of several pteridine metabolic genes. In *Leishmania*, resistance to the antifolate methotrexate is mediated through several mechanisms singly or in combination, including alterations in transport leading to reduced drug influx, overproduction (R-region amplification) or point mutation of dihydrofolate reductase-thymidylate synthase (*DHFR-TS*), and amplification of a novel pteridine reductase (*PTR1*, encoded by the H-region). All of the proteins involved are potential targets for antifolate chemotherapy. Notably, parasites in which the gene encoding dihydrofolate reductase (DHFR) has been deleted (*dhfr-ts*$^-$ knockouts) do not survive in animal models, validating this enzyme as a target for effective chemotherapy. However, the properties of pteridine reductase 1 (PTR1) suggest a reason why antifolate chemotherapy has so far not been successful in trypanosomatids. PTR1, by its ability to provide reduced pterins and folates, has the potential to act as a by-pass and/or modulator of DHFR inhibition under physiological conditions. Moreover, PTR1 is less sensitive to many antifolates targeted primarily against DHFR. These findings suggest that successful antifolate chemotherapy in *Leishmania* will have to target simultaneously both DHFR and PTR1.

Key words: *Leishmania*, pteridine reductase, chemotherapy, drug resistance, methotrexate.

INTRODUCTION

Leishmania are trypanosomatid protozoan parasites transmitted by phlebotomine sand flies that infect over 12 million people worldwide. Leishmaniasis manifests as minor or severe cutaneous lesions, or as a visceral form which if untreated has a fatality rate of 100%. Its emergence as an opportunistic pathogen in AIDS patients (Olliaro & Bryceson, 1993) has further raised the public health significance of leishmaniasis and the need to control this disease. Treatment is based on pentavalent antimonial compounds, but unfortunately these drugs are frequently toxic, have an unknown mode of action and are often only marginally effective. The problem of chemotherapy is further compounded by the development of drug resistance in various endemic regions of the world (Jackson, 1990; Grogl, Thomason & Franke, 1992). No effective vaccine against leishmaniasis is available.

* Corresponding author: Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, 240 Longwood Avenue, Boston, MA 02115. Tel: (617) 432-0611. Fax: (617) 432-1476. E-mail: sbeverle@warren.med.harvard.edu.

A better understanding of the biochemistry of *Leishmania*, particularly pathways unique to the parasite, would facilitate our capacity to devise rational pharmacological strategies for treatment. One metabolic pathway that has been successfully exploited/targeted for the treatment of several parasitic diseases involves the biosynthesis of reduced folate co-factors. However, classical inhibitors of folate biosynthesis (e.g. sulphonamides, trimethoprim and pyrimethamine) are ineffective against *Leishmania* (Mattock & Peters, 1975; El On *et al.* 1984; Neal & Croft, 1984). This probably reflects the fact that pteridine metabolism is rather unusual in *Leishmania*. Here, we review recent advances in the understanding of pteridine (folate and pterin) metabolism in *Leishmania*. We focus on the properties of pteridine reductase 1 (PTR1), a novel *Leishmania* enzyme responsible for salvage of pteridines and resistance to the antifolate drug methotrexate (MTX) (Callahan & Beverley, 1992; Papadopoulou, Roy & Ouellette, 1992; Bello *et al.* 1994; Papadopoulou *et al.* 1994*b*) We contrast the properties and metabolic roles of PTR1 to those of dihydrofolate reductase-thymidylate synthase (DHFR-TS), the main target for MTX, and discuss

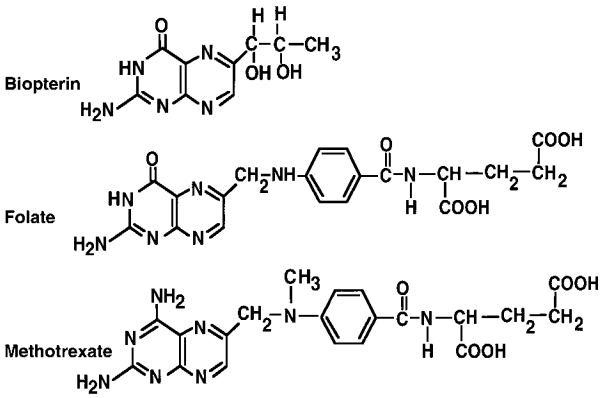

Fig. 1. Pteridine structures. Biopterin is a pterin, which are also referred to as unconjugated pteridines. Folate is classified as a conjugated pteridine.

how PTR1 plays a significant role in the biology of the parasite and in modulating the sensitivity of *Leishmania* to antifolate inhibition of DHFR-TS. These findings provide some insights into new strategies for antifolate chemotherapy against *Leishmania*. We also discuss the use of *PTR1* overexpression and deletion as a tool to manipulate endogenous reduced pterin pools, which has lead to new insights into the role of pterins in *Leishmania*.

GROWTH REQUIREMENTS FOR PTERINS AND FOLATES

Leishmania and related trypanosomatid protozoans lack a *de novo* pathway for the biosynthesis of pteridines and are thought to rely exclusively on salvage of both pterins and folates (see Fig. 1 for structures). The requirement for pteridines in growth of trypanosomatids was first revealed by the existence of '*Crithidia* factor', required for growth of *Crithidia fasciculata*, a trypanosomatid protozoan related to *Leishmania* (Nathan & Cowperthwaite, 1955; Nathan, Hutner & Levin, 1956; Kidder & Dutta, 1958). *Crithidia* factor was shown to be biopterin, an unconjugated pteridine (Fig. 1), and its discovery ultimately led to an understanding of its metabolic role in eukaryotes (Kaufman, 1963; Tietz, Lindberg & Kennedy, 1964; Tayeh & Marletta, 1989). Subsequent studies of several *Leishmania* species have shown that these parasites have an absolute requirement for some form of pteridine, and that biopterin and a number of related unconjugated pterin analogues can reduce or even eliminate the requirement for folic acid in the trypanosomatid growth media (Trager, 1969; Scott, Coombs & Sanderson, 1987; Kaur *et al*. 1988; Petrillo-Peixoto & Beverley, 1988*a*; Beck & Ullman, 1990; Bello *et al*. 1994; Papadopoulou *et al*. 1994*b*).

A significant experimental caveat is posed by the use of 'defined' media: folate and biopterin are required in such minute quantities that even trace contaminants can yield growth in supposedly pteridine-free media (Kaur *et al*. 1988; Petrillo-Peixoto & Beverley, 1988*a*). Moreover, folates can decompose and yield unconjugated pterins. In recognition of this limitation, we and others generally use the term folate- or pterin-'deficient' in referring to 'defined' media.

UPTAKE AND SALVAGE OF PTERINS AND FOLATES

As *Leishmania* are pteridine auxotrophs, they possess a complete and sophisticated pathway for salvaging pteridines from the host and incorporating them into intermediary metabolism. Several studies have shown the existence of specific high affinity transporters for folates in *Leishmania* (Ellenberger & Beverley, 1987*a*, *b*; Beck & Ullman, 1989), and recent work has revealed the existence of a transporter for unconjugated pteridines as well (J. Moore & S. M. Beverley, unpublished). Promastigotes of *L. donovani* are capable of incorporating biopterin into folate *in vitro* (Beck & Ullman, 1991), suggesting the possibility of a *de novo* folate biosynthetic pathway. In contrast, *L. major* promastigotes fail to incorporate para-aminobenzoic acid into folate (Kovacs *et al*. 1989), and most sulphonamides fail to inhibit the growth of *L. major* or *L. donovani* (Kaur *et al*. 1988; Petrillo-Peixoto & Beverley, 1988*a*). The effects of even those which are active are not reversed by exogenous thymidine, which nonetheless can reverse MTX inhibition (Kaur *et al*. 1988; Petrillo-Peixoto & Beverley, 1988*a*; B. Nare & S. M. Beverley, unpublished results). These data suggest that the folate synthetic pathway, if present, differs considerably from the classical pathway through dihydropteroate, synthase (Hitchings & Burchall, 1965; Hitchings, 1978).

We discuss below the properties of PTR1, the reductase which permits *Leishmania* to incorporate internalized pterins into the parasite's metabolic pathways. In this regard *Leishmania* differs considerably from the host cell, which can synthesize pterins *de novo* from GTP in the form of dihydroneopterin and ultimately tetrahydrobiopterin (H_4biopterin) (Nichol, Smith & Duch, 1985). Since mammalian cells synthesize reduced pterins exclusively, they have no need for an enzyme specialized in oxidized pterin salvage.

MECHANISMS OF ANTIFOLATE RESISTANCE

Selection of *Leishmania* for resistance to MTX, a folate antagonist (Fig. 1), leads to the development of various forms of resistance such as reduced drug uptake, gene amplification, and/or structurally altered targets (Fig. 2) (Beverley *et al*. 1984; Ellenberger & Beverley, 1987*a*, 1989; Petrillo-Peixoto & Beverley, 1988*a*; White *et al*. 1988; Beck & Ullman, 1989; Beverley, 1991; Callahan &

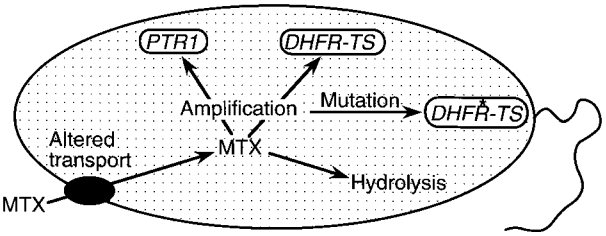

Fig. 2. Mechanisms for methotrexate resistance in *Leishmania*. Resistance to the antifolate methotrexate (MTX) can arise from a variety of mechanisms. Alterations in transport can lead to decreased uptake of MTX and folates (and possibly pterins). Amplification of the gene encoding DHFR-TS can overwhelm intracellular MTX inhibition, while amplification of the gene encoding PTR1 provides an alternative path for synthesizing reduced folates (Figs 3, 4). PTR1 is much less sensitive to MTX than DHFR-TS (Table 1). A point mutation leading to a MTX-insensitive DHFR-TS has been reported, albeit in the context of gene amplification. Potentially, increased levels of MTX/folate hydrolysing enzymes could yield resistance, although this has not been observed so far.

Beverley, 1992; Papadopoulou, Roy & Ouellette, 1992; Arrebola *et al*. 1994; Gamarro *et al*. 1994; Segovia, 1994; Borst & Ouellette, 1995; Gueiros-Filho & Beverley, 1996). These studies have provided much of the knowledge about drug resistance, mutagenesis and pteridine metabolism in *Leishmania*. Moreover, the amplified DNAs have been turned successfully into vectors for stable DNA transfection and expression, which have proven valuable in many subsequent studies (Kapler, Coburn & Beverley, 1990; LeBowitz *et al*. 1990).

Overproduction or mutation of DHFR-TS

Certain MTX-resistant *Leishmania* show amplification of the R-region, which contains the gene (*DHFR-TS*) encoding DHFR-TS. This enzyme is the presumptive primary cellular target for MTX (Figs 2, 3) (Coderre *et al*. 1983; Beverley *et al*. 1984; Beverley, Ellenberger & Cordingley, 1986; Beverley, 1991). Overproduction of DHFR-TS in response to MTX pressure is analogous to the amplification of mono-functional DHFR observed in mammalian MTX-resistant lines (Schimke, 1984, 1988). Less commonly, point mutations in *DHFR-TS* leading to an antifolate-resistant enzyme have been observed (Arrebola *et al*. 1994). As in mammalian cells, this has only been seen in the context of simultaneous gene amplification, perhaps because the enzyme is less catalytically active than the wild-type (Haber *et al*. 1981). In contrast, point mutation in *DHFR-TS* is the predominant mechanism of clinical antifolate resistance in *Plasmodium falciparum*, whereas laboratory selections have shown both point mutations and amplifications (Hyde, 1990; Wellems, 1991; Watanabe & Inselburg, 1994).

H-region amplification and the discovery of PTR1

Another DNA amplification was observed in lines showing *DHFR-TS* amplification, termed the H region (Fig. 2; Beverley *et al*. 1984). Remarkably, amplification of this region was also observed following selection of *Leishmania* with MTX or other structurally and mechanistically unrelated drugs such as terbinafine, primaquine or arsenite (Beverley *et al*. 1984; Hightower *et al*., 1988; Detke, Katakura & Chang, 1989; Ellenberger & Beverley, 1989; Katakura & Chang, 1989; Ouellette *et al*. 1991; Papadopoulou, Roy & Ouellette, 1993; Papadopoulou *et al*. 1994*a*). This raised the question as to whether a single or multiple gene in this 40 kb region was responsible for drug resistance. Northern blot studies showed that the *L. major* H-region encoded at least 19 polyadenylated mRNAs (Ellenberger, 1989). Interestingly, unselected laboratory stocks of the lizard parasite *L. tarentolae* bear spontaneous amplifications of the H-region (Petrillo-Peixoto & Beverley, 1988*b*; White *et al*. 1988), suggesting that this amplicon encodes gene(s) that could have other roles in *Leishmania*.

Transfection studies showed that the H-region contained two different drug resistance genes. *PTR1* encodes a broad spectrum pteridine reductase (PTR1) mediating resistance to MTX and primaquine, which is discussed extensively below. *PGPA*, a member of the ABC family of membrane transporters belonging to the *MRP* subfamily of the P-glycoproteins (Cole *et al*. 1992; Ouellette, Legare & Papadopoulou, 1994), was shown to be responsible for arsenite resistance encoded by the H-region (Callahan & Beverley, 1991; Papadopoulou *et al*. 1994*a*). *PGPA*-mediated resistance is often referred to as 'low-level' resistance since much stronger arsenite resistance loci are found in some *Leishmania* lines (Papadopoulou *et al*. 1994*a*). However, in *L. major* certain alleles of *PGPA* confer at least 12-fold resistance (Callahan & Beverley, 1991), which is greater than that conferred by overexpression of the P-glycoprotein MDR1 in *Leishmania* (Chow *et al*. 1993). PTR1 and PGPA are currently thought not to mediate cross-resistance to their respective cognate drugs, indicating that the collateral drug resistances conferred by H-region amplification arise through a 'hitch-hiking' effect.

Alterations in transport or metabolism

Another way that *Leishmania* resist the toxic effects of MTX is through reduced uptake, which is paralleled by decreased folate uptake since these two pteridines enter the cell predominantly through the same transport system (Fig. 2; Ellenberger & Beverley, 1987*b*; Kaur *et al*. 1988). Several lines exhibiting total or partial loss of MTX transport activity have been described in *L. donovani*, *L. major*

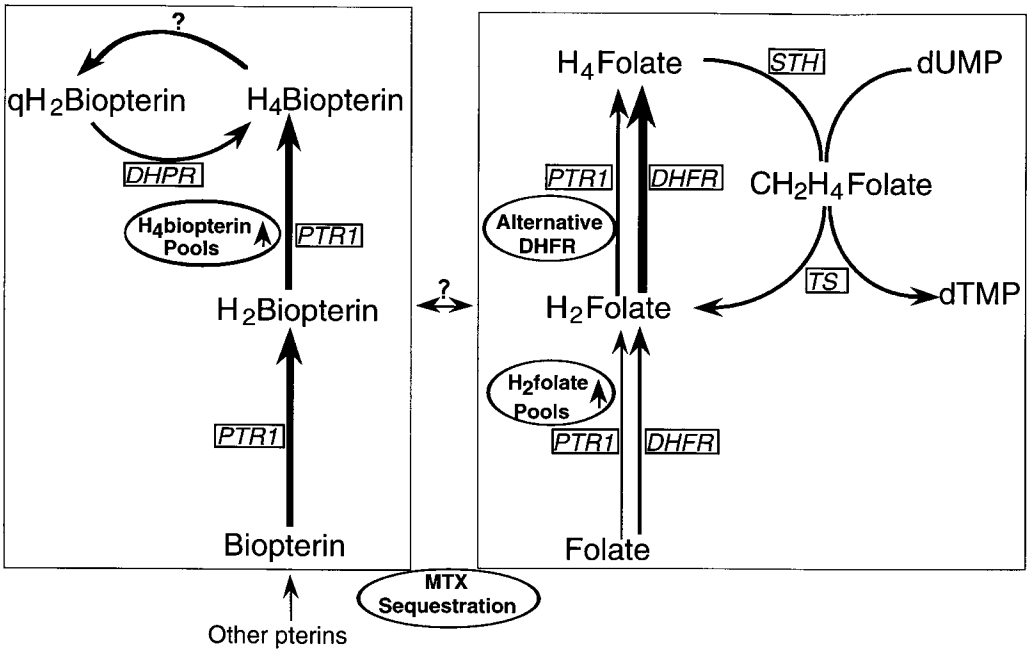

Fig. 3. Pteridine metabolic pathways in *Leishmania*. For PTR1 and DHFR, the size of arrows corresponds to the relative contribution of each enzyme. PTR1, pteridine reductase 1; DHFR, dihydrofolate reductase; TS, thymidylate synthase; STH, serine transhydroxymethylase; CH_2-H_4folate, 5,10-methylene tetrahydrofolate; DHPR, dihydropteridine reductase; ?, unknown enzyme/metabolic function. Sites where PTR1 has been (or proposed to be) implicated in MTX resistance are indicated by shaded circles. First, PTR1's folate reductase and H_2folate reductase activity provide an alternative MTX-refractory DHFR activity. Second, PTR1 overexpression could increase H_2folate pools, thereby relieving MTX inhibition of DHFR-TS directly. Third, overexpressed PTR1 could bind and sequester MTX. Lastly, by increasing H_4biopterin pools, PTR1 could alleviate MTX induced toxicity arising from inhibition of pterin metabolism directly.

and *C. fasciculata* (Dewes, Ostergaard & Simpson, 1986; Ellenberger & Beverley, 1987*b*; Kaur *et al.* 1988; Beck & Ullman, 1989). Interestingly, the MTXA5 mutant of *L. donovani* shows collateral phenotypes, such as an inability to grow solely on biopterin (Beck & Ullman, 1989, 1990). This trait maps to the same locus as the MTX-transport defect in reversion assays (Beck & Ullman, 1990) and has been used in our laboratory to isolate several loci mediating MTX and/or folate transport by cosmid rescue (J. Moore and S. M. Beverley, unpublished). Ultimately, an understanding of folate transport in modulating antifolate sensitivity and uptake of antifolates themselves will be important in the design of potential drugs.

Leishmania and other trypanosomatids possess MTX-hydrolyzing enzymes (Oe, Kohashi & Iwai, 1984; Kaur *et al.* 1988; Ellenberger *et al.* 1989), which could potentially mediate resistance to conjugated pteridine inhibitors (such as MTX; Fig. 1) if overproduced (Fig. 2). This seemingly attractive drug resistance mechanism has not been encountered so far.

PTR1 AND PTERIDINE METABOLISM

PTR1 was identified as the H-region MTX resistance gene, and was originally named $HMTX^R$ in *L. major* (Callahan & Beverley, 1992) or *LTDH* in *L. tarentolae* (Papadopoulou, Roy & Ouellette, 1992). Searches of protein databases with the PTR1 amino acid sequence revealed homology with short chain dehydrogenase/reductase (SDR) and aldo-keto reductase (AKR) families (Callahan & Beverley, 1992; Papadopoulou, Roy & Ouellette, 1992), both of which contain a large number of enzymes with diverse substrates. The SDR family includes several enzymes involved in pteridine metabolism, such as sepipaterin reductase and dihydropteridine reductase (Krozowski, 1994; Jornvall *et al.* 1995), suggesting a potential role for PTR1 in pterin/folate metabolism. However, the sequence relationships are distant (25% identity or less), and other enzymes with non-pteridine substrates are more closely related to PTR1. Thus, we used biochemical and genetic approaches to determine its role (Bello *et al.* 1994; Papadopoulou *et al.* 1994*b*; Luba *et al.* (unpublished); Nare, Hardy & Beverley, 1997).

Enzymatic properties of PTR1

Leishmania major PTR1 was expressed in *Escherichia coli* using the T7 system (Studier *et al.* 1990), and purified to homogeneity through a combination of ion-exchange and gel filtration on fast protein liquid chromatography (Bello *et al.* 1994; Nare, Hardy & Beverley, 1997). Analogous purification schemes

Table 1. Comparison of the biochemical properties of PTR1 and DHFR-TS

Property	*L. major* PTR1	*L. major* DHFR-TS
Molecular weight		
Subunit	30000	56000
Oligomeric state	Homotetramer	Homodimer
Cofactor	NADPH (K_m = 12 μM)	NADPH (K_m = 3 μM)
Substrates	Pterins – pH 4·7 (K_m = 10 μM)	Folate – pH 6·0 (K_m = 4 μM)
	Folates – pH 6·0 (K_m = 2·5 μM)	H_2-folate – pH 7·0 (K_m = 2 μM)
Reaction sequence	Ordered sequential	Random
MTX-inhibition	Biopterin (K_i = 30 nM)	
	H_2-folate (K_i = 190 nM)	H_2-folate (K_i = 0·13 nM)

Summarized from Bello *et al*. 1994; Nare, Hardy & Beverley, 1997; Luba *et al*. unpublished; Meek, Garvey & Santi, 1985.

were used to obtain homogeneous native *L. major* and recombinant *L. tarentolae* PTR1s. All PTR1s behave similarly, and the active enzyme consists of a tetramer of 30 kDa subunits (Nare, Hardy & Beverley, 1997).

PTR1 displayed biopterin reductase and folate reductase activity in the presence of NADPH but not NAPH, indicating that PTR1 is a broad substrate pteridine reductase (Table 1; Bello *et al*. 1994; Nare, Hardy & Beverley, 1997). The pH optimum for activity towards biopterin was 4·7 while that for folate was 6·0. Dihydrobiopterin (H_2biopterin) and dihydrofolate (H_2folate) were also reduced with optimum pHs similar to those observed with the respective oxidized forms. The K_m for the NADPH was similar for both folates and biopterin substrates. However, folates had lower K_m values than biopterins at all pHs (Table 1), indicating intrinsic differences in their interactions with PTR1. H_2biopterin and H_2folate as well as other dihydropteridines (H_2pteridines) displayed substrate inhibition at higher concentrations, a phenomenon not observed with oxidized pteridines (Nare, Hardy & Beverley, 1997). Although its physiological relevance is unknown, current data suggest that substrate inhibition could arise from the presence of dead-end binary complexes between PTR1 and some substrates, including H_2folate (Luba *et al*. unpublished). Studies of such dead-end complexes may provide leads for developing inhibitors. PTR1 generates tetrahydropteridines when provided with either fully oxidized or H_2pteridines and H_2folate has been shown to be a transient intermediate in the conversion of folate to H_4folate (Luba *et al*. unpublished; Nare, Hardy & Beverley, 1997). PTR1 was predicted to be a MTX target (Callahan & Beverley, 1992), and indeed the enzyme was inhibited by MTX with all pteridine substrates tested (Table 1; Bello *et al*. 1994; Nare, Hardy & Beverley, 1997).

Comparison of PTR1 with other pteridine reductases

The enzymatic properties of PTR1 clearly establish the distinctiveness of this enzyme from other previously described pteridine reductases such as dihydropteridine reductase (DHPR) and DHFR (Bello *et al*. 1994; Luba *et al*. unpublished; Nare, Hardy & Beverley, 1997). Stereochemical studies have shown that PTR1, like DHPR, is a B-side dehydrogenase, and in combination with site-directed mutagenesis studies confirms the membership of PTR1 in the SDR family (Krozowski, 1994; Luba *et al*. unpublished data). In contrast, the AKR family members, to which PTR1 also shows some similarities, are A-side dehydrogenases.

PTR1 lacks the quininoid-H_2biopterin reductase activity of DHPR, and *Leishmania* has a separate DHPR activity unrelated to PTR1 (Bello *et al*., 1994). Instead, the activities of PTR1 overlap with those of DHFRs from various sources, which are known to reduce both folates and H_2pterins (Webber & Whiteley, 1985; Smith *et al*. 1987). DHFRs have no sequence similarity to PTR1 or other members of the SDR family.

Since the properties of the *Leishmania* DHFR-TS with folate or unconjugated pteridines had not been examined, we purified homogeneous recombinant *L. major* DHFR-TS by MTX affinity chromatography. DHFR-TS displayed high activity with H_2folate (Meek, Garvey & Santi, 1985), poor activity towards folate, and no detectable activity with pterin substrates including biopterin and H_2biopterin (Table 1; Nare, Hardy & Beverley, 1997). Thus, unlike mammalian DHFRs which possess activity towards H_2pteridines including H_2biopterin (Webber & Whiteley, 1985; Smith *et al*. 1987), *Leishmania* DHFR lacks this activity. Presumably, H_2biopterin reductase activity arising from PTR1 is sufficient to provide *Leishmania* with its cellular requirements for fully reduced biopterin. The availability of both *dhfr-tr*$^-$ (Cruz & Beverley, 1990) and *ptr1*$^-$ (Bellow *et al*. 1994) mutants permitted direct assessment of the contribution of each enzyme to activity in crude extracts. These experiments showed that PTR1 contributed about 10% of total folate reduction in wild-type *Leishmania*, and, of course, all of the unconjugated pterin reductase activity (Nare, Hardy & Beverley, 1997).

Table 2. *Leishmania* growth in 'pteridine-depleted' media

Supplements	Wild Type	*ptr1*$^-$	*ptr1*$^-$*1* +PTR1
None	−	−	−
Biopterins			
Oxidized biopterin	+	−	+
Reduced biopterin	+	+	+
Other oxidized pterins			
Good PTR1 substrates	+	−	+
Poor PTR1 substrates	−	−	+
Non-PTR1 substrates	−	−	−

The *ptr1*$^-$ mutant was generated by double gene replacement, and *PTR1* was re-introduced into this line to give *ptr1*$^-$*1+PTR1* which shows 100-fold overexpression of PTR1 (Bello *et al*. 1994). PTR1 activity with various pterins was measured at pH 4·7 (Nare, Hardy & Beverley, 1997). (+) Growth observed for at least 6 continuous passages. (−) No growth observed.

The growth requirements of PTR1 mutants suggest a key role in pterin salvage

The properties of PTR1 suggested an essential role in the salvage of pteridines (Bello *et al*. 1994) and subsequent experiments have confirmed this notion. First, *ptr1*$^-$ null mutants require reduced pterins (H_2- or H_4biopterin) for growth in culture, a requirement not satisfied by oxidized pterins (Table 2), oxidized or reduced folates or thymidine (or combinations thereof). Second, the ability of *L. major* to grow in a broad range of pterin substrates correlated with their ability to serve as PTR1 substrates. Good PTR1 substrates sustained growth of wild type *Leishmania* but poor substrates required the overexpression of the enzyme in order to support parasite growth (Table 2). The spectrum of pterins capable of supporting growth of *L. major* (Bello *et al*. 1994; Nare, Hardy & Beverley, 1997) was identical to that observed with *L. donovani* (Beck & Ullman, 1990). Similarly, *ptr1*$^-$ mutants and overexpressing lines of *L. tarentolae* (Papadopoulou *et al*. 1994*b*) behave like those of *L. major*. Thus, the role of PTR1 in pterin salvage appears to be conserved in all *Leishmania* species. In contrast to PTR1, the role of DHFR-TS is limited to the maintenance of reduced folate pools, since DHFR-TS lacks pterin reductase activity and its absence (*dhfr-ts*$^-$) or overproduction does not alter the pterin-dependent growth profile of *Leishmania* (Nare, Hardy & Beverley, 1997).

What is the role of pterins in Leishmania*?*

Since the *ptr1*$^-$ knockout *L. major* are rescued by reduced pterins, but not reduced folates, it follows that reduced pterins must play an essential role. Remarkably, this role(s) has yet to be established. In humans, H_4biopterin is a co-factor for aromatic amino acid hydroxylations (Kaufman, 1963) leading to biosynthesis of important neurotransmitters, and defects in biopterin biosynthesis leads to serious neurological disorders. Cleavage of ether-linked lipids (Tietz, Lindberg & Kennedy, 1964) and the biosynthesis of nitric oxide (Tayeh & Marletta, 1989) also require H_4biopterin as cofactor. So far, none of the known functions of H_4biopterin have been detected in trypanosomatids. For example, *C. fasciculata* cannot convert phenylalanine to tyrosine (Kidder & Dewey, 1963), and the *Leishmania* ether-linked lipid cleavage activity is NADPH-dependent (Ma, Beverley & Turco, 1996). Other potential roles, such as in the synthesis of molybdopterins or in NO biosynthesis, have not been studied.

PTR1 and oxidant resistance

The ability to genetically manipulate reduced pteridine levels in *Leishmania* through deletion or overexpression of PTR1 provides a powerful tool for probing pterin function *in vivo*. This is exemplified by our studies of a *Leishmania* line (PQR30) selected for resistance to primaquine (Ellenberger & Beverley, 1989). PQR30 exhibited H-region amplification, and subsequent studies showed that PTR1 was the gene responsible for primaquine resistance (Bello *et al*. 1994). However, primaquine was not a substrate for PTR1 activity. Primaquine is known to induce oxidative stress (Augusto *et al*. 1986), and reduced pteridines are known to be highly reactive with reactive oxygen intermediates (ROI) (Heales *et al*. 1988; Kojima *et al*. 1992, 1995; Shen & Zhang, 1993; Kurobane *et al*. 1995). Thus, we proposed that PTR1 was involved in ROI resistance through the provision of reduced pteridines *in vivo*. Subsequent tests with *ptr1*$^-$ knockouts and overexpressing lines showed that PTR1 overexpressors are resistant to a variety of reactive ROIs, while *ptr1*$^-$ knockouts were hypersensitive, and that this effect was pterin-dependent (B. Nare, L. A. Garraway & S. M. Beverley, unpublished results). The molecular mechanism by which reduced pterins mediate ROI resistance *in vivo* is under investigation.

These data suggest a new biological role for pterins in *Leishmania*, one potentially relevant to higher eukaryotes as well. It also has implications to the survival of the parasite within the host, since macrophages are able to induce varying degrees of oxidative stress depending upon their activation state. Potentially, inhibitors of PTR1 could decrease the capacity of *Leishmania* to survive host-induced oxidative stress, as do many antiparasite agents.

In summary, characterization of the enzymatic and metabolic roles of PTR1 and DHFR-TS has yielded a comprehensive model of pteridine metabolism in *Leishmania* (Fig. 3; Bello *et al*. 1994; Nare, Hardy & Beverley, 1997). This framework allows us to test hypotheses about pteridine metabolism, to

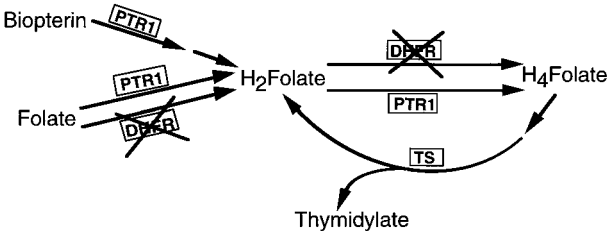

Fig. 4. Model for how PTR1 comprises DHFR-directed chemotherapy. In this model, the primary cellular target of antifolates is considered to be DHFR-TS. MTX inhibition (indicated by an 'X') deprives *Leishmania* of reduced folate co-factors. However, PTR1, which is less sensitive to MTX, alleviates or 'by-passes' this blockage by synthesizing reduced H_4folate from H_2folate or folate. For the parasite in the mammal, PTR1 may also permit salvage of host folates which would further compromise MTX inhibition.

evaluate mechanisms of MTX resistance, to generate new ideas to study pterin functions, and to develop new strategies for antifolate chemotherapy.

THE ROLE OF PTR1 IN MODULATING MTX SENSITIVITY AND RESISTANCE

Susceptibility of *Leishmania* to antifolates is significantly modulated by exogenous folates (Kaur *et al.* 1988; Petrillo-Peixoto & Beverley, 1988*a*; B. Nare & S. M. Beverley, unpublished results) and the ability of PTR1 to synthesize reduced folates readily accounts for this (Figs 3, 4) For example, *ptr1*$^-$ *Leishmania* are highly sensitive to MTX (Bello *et al.* 1994; Papadopoulou *et al.* 1994*b*), and folate modulation of MTX toxicity is dramatically decreased as well (B. Nare & S. M. Beverley, unpublished results). Mammalian cells, which lack alternative means to reduce folates, display very little ability to modulate MTX toxicity when provided with exogenous folates (Oe, Kohashi & Iwai, 1983; MacDonald & Bode, 1988). It seems likely that PTR1-mediated modulation of MTX potency may play an important role in antileishmanial chemotherapy *in vivo*. For example, antifolates were effective against *Leishmania* in cultured macrophages only when cultivated in 'folate-free' media (Sirawaraporn *et al.* 1988).

In this light, it is reasonable to ask whether the folate pathway is even a good target for chemotherapy at all. This has been clearly answered by studies of the *dhfr-ts*$^-$ knockout *L. major* (Cruz & Beverley, 1990). These parasites require thymidine for growth, and fail to survive in macrophages *in vitro* or in animal infections, including those of susceptible BALB/c and nude mice (Titus *et al.* 1995). Importantly, provision of thymidine in these studies resulted in rescue of parasite growth and pathology, showing that it is loss of *dhfr-ts* alone that causes *Leishmania* to perish. Thus, the use of genetic knockouts have clearly validated DHFR-TS as a target for antiparasite chemotherapy. Similar studies of the *ptr1*$^-$ knockouts are underway in our laboratory.

PTR1 and MTX resistance

The properties of PTR1 provide a clear understanding of the forces leading to its amplification following MTX selection. PTR1 is much less sensitive to MTX than DHFR-TS (Table 1; Bello *et al.* 1994; Nare, Hardy & Beverley, 1997). This allows PTR1 to provide a metabolic by-pass of the blocked DHFR-TS at most MTX concentrations (Fig. 3). However, PTR1 is expressed at very low levels (0·01% of cellular protein) and only provides about 10% of the total cellular folate-reducing activity (Nare, Hardy & Beverley, 1997). Thus, overexpression of PTR1 (via H-region amplification) is necessary in order to provide sufficient activity under MTX pressure. Although this is the presently favoured mechanism, others exist which may act simultaneously. PTR1 could mediate MTX resistance via its ability to reduce folate to generate H_2folate, which is known to be very effective in relieving inhibition of DHFR-TS by MTX *in vitro* (White, 1979), or through some mechanism arising from its ability to reduce biopterin or other pterins (Fig. 3).

Implications of PTR1 for antifolate chemotherapy

Given that DHFR-TS (and perhaps PTR1 as well) represents a valid drug target, but one potentially compromised by the activity of PTR1 (Figs 3, 4), we propose that it will be necessary to inhibit simultaneously both of these enzymes in order to develop effective chemotherapy. This could require the development of two separate compounds, targeted separately against DHFR and PTR1. Given the structural overlap amongst the substrates of these two enzymes, it is conceivable that a single compound might be found with good efficacy against both enzymes. Recently, we have in fact identified a lead compound which shows good potency against both DHFR and PTR1 and which shows good efficacy against promastigotes in culture and amastigotes in cultured macrophages (B. Nare & S. M. Beverley, unpublished). This suggests that combined inhibition is an attainable goal. Unfortunately, our lead compound has no activity in animal infection models, perhaps because of problems involving drug absorption and/or metabolism within the host. This illustrates a widely recognized pharmacological dictum, that 'inhibitors are not the same as drugs'. Nonetheless, we are optimistic about embarking upon a search for new prospective drugs. This will be aided by the availability of the three-dimensional structure of *L. major* DHFR-TS (Knighton *et al.* 1994), and we are currently pursuing structural

studies of PTR1. Thus, as with other protozoan parasites, pteridine metabolism may yet prove to be a feasible target for chemotherapy in *Leishmania*.

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