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Biochemical and Genetic Tests for Inhibitors of *Leishmania* Pteridine Pathways

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Hardy, L. W., Matthews, W., Nare, B., and Beverley, S. M. 1997. Biochemical and genetic tests for inhibitors of *Leishmania* pteridine pathways. *Experimental Parasitology* **87,** 158–170. The study of antifolate-resistant mutants of the protozoan parasite *Leishmania* has provided useful information about genetic processes such as gene amplification and mutation and knowledge of the unique features of the pteridine metabolic pathway in this primitive eukaryote. The novel bifunctional dihydrofolate reductase–thymidylate synthase (DHFR-TS) is an essential enzyme, yet most DHFR-TS inhibitors show little promise as potential drugs. *Leishmania* possess a novel alternative pteridine reductase (PTR1) which is relatively insensitive to methotrexate. We have proposed that the ability of PTR1 to serve as a metabolic bypass and thus modulate drug inhibition of DHFR-TS activity may be responsible for the poor efficacy of many antifolates. In this work, we have sought inhibitors of *L. major* PTR1 from a collection of 74 compounds. The most potent inhibitors were also tested against *L. major* DHFR-TS and human DHFR and several compounds showing good activity for PTR1 alone, or for all three reductases, were identified. The activity of these compounds was tested against wild-type promastigotes, and those which were potent inhibitors of both PTR1 and DHFR-TS (but not those active against only PTR1) showed good potencies. Growth inhibition tests of *L. major* mutants, lacking PTR1 or DHFR-TS (*ptr1*$^-$ and *dhfr-ts*$^-$ knockouts) or overexpressing PTR1, were used as a ''genetic screen'' to assess whether these two pteridine reductases were targets *in vivo.* Remarkably, only one compound showed a methotrexate-like pattern of inhibition. Six compounds showed good inhibition of *Leishmania* growth regardless of PTR1 or DHFR-TS levels. These findings suggest that *Leishmania* cells contain multiple targets for a diverse set of antifolates, with one or more significant targets in addition to DHFR-TS and PTR1. This emphasizes the necessity of combined biochemical and genetic screens in efforts to rationally design chemotherapeutic strategies in *Leishmania.*

Index Descriptors and Abbreviations: antifolates; diaminopyrimidines; dihydrofolate reductase (EC 1.5.1.3); drug resistance; engineered mutants; enzyme inhibitors; *Leishmania;* methotrexate; pteridine metabolism; pteridine reductase (new enzyme for which the EC number is not yet assigned; an application/request has been submitted); pterin analogs; quinazolines; thymidylate synthase (EC 2.1.1.45); DHFR, dihydrofolate reductase; DHFR-TS, dihydrofolate reductase–thymidylate synthase; MTX, methotrexate; PTR1, pteridine reductase 1.

INTRODUCTION

Leishmania exhibit many unusual features in pteridine metabolic pathways which are essential for growth, suggesting that these should be excellent targets for chemotherapeutic attack (summarized in Nare *et al.* 1997a). However, unlike some other protozoal infectious diseases where antifolates such as pyrimethamine, sulfa drugs, and trimethoprim have enjoyed success (Ferone 1984; Grossman and Remington 1979; McDougald 1982), effective antifolate chemotherapy has not been achieved in infections by *Leishmania,* despite this parasite's extreme divergence from the host. Thus, we and other laboratories have studied antifolate-resistant mutants to identify genes and processes impli-

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cated in antifolate resistance and as a tool to probe these important pathways. Thus far, two potential targets of antifolate action have been identified: the first was the bifunctional dihydrofolate reductase–thymidylate synthase (DHFR-TS), which is the primary source of reduced folates and the only source of thymidylate in the parasite (Nare *et al.* 1997b; Cruz and Beverley 1990; Ivanetich and Santi 1990; Coderre *et al.* 1983). The second was a novel broad spectrum reductase, pteridine reductase 1 or PTR1, which is required for the essential salvage of unconjugated pterins such as biopterin (Bello *et al.* 1994; Nare *et al.* 1997b). The reactions catalyzed by the DHFR domain of DHFR-TS and by PTR1 are shown in Fig. 1. Either or both of the *PTR1* and *DHFR-TS* genes are often found to be amplified in methotrexate (MTX) resistant lines (Beverley *et al.* 1984; reviewed in Beverley 1991 and Borst and Ouellette 1995). Another form of resistance is exemplified by decreased MTX uptake (Ellenberger and Beverley 1987b; Dews *et al.* 1986; Kaur *et al.* 1988). These forms of resistance can occur singly or in various combinations.

Investigations of the genes and enzymes identified by studies of antifolate resistance have provided important beginnings for efforts to develop effective antifolate chemotherapy and to understand why previously tested inhibitors did not work. This is exemplified by studies of the PTR1 enzyme, which contributes a sig-

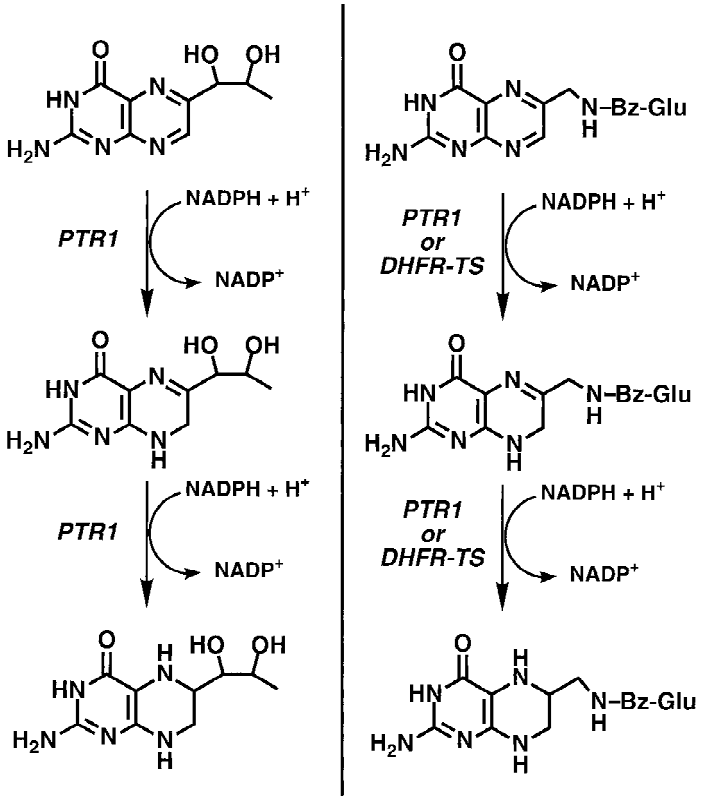


FIG. 1. Reactions catalyzed by the DHFR domain of DHFR-TS and by PTR1 from *Leishmania major*. The left side depicts the reduction of biopterin to 5,6,7,8-tetrahydrobiopterin. The right side depicts the reduction of folate to 5,6,7,8-tetrahydrofolate. Also shown are the reductions of the dihydro compounds, which are intermediates in the conversion of the fully oxidized to the fully reduced pteridines. Bz-Glu represents the *N*-(benzoyl)-L-glutamate portion of folate. PTR1 catalyzes the NADPH-dependent reduction of a broad range of pterins, in addition to the reactions depicted here (Nare *et al.* 1997a).

nificant portion of the total dihydrofolate reductase activity in *Leishmania* (Nare *et al.* 1997a). *Leishmania ptr1*$^{-}$ knockout mutants exhibit hypersensitivity to MTX (Bello *et al.* 1994; Papadopoulou *et al.* 1994), and under physiological conditions, PTR1 activity has been observed to significantly reduce the sensitivity of *Leishmania* to antifolates (Nare and Beverley, manuscript in preparation). Thus, PTR1 acts as both a modulator of and a metabolic ''bypass'' for inhibitors targeting DHFR-TS (Bello *et al.* 1994; Nare *et al.* 1997a,b). Notably, PTR1 is considerably less sensitive than DHFR-TS to antifolates such as MTX. This MTX-insensitive, alternative reductase thus poses a considerable challenge to strategies targeting DHFR-TS exclusively. It should also be stressed that deletion of *PTR1* is lethal unless an alternative source of its metabolic products (reduced biopterin) is provided, showing that PTR1 is potentially a drug target in its own right (Nare *et al.* 1997a).

Thus, we have proposed that successful antifolate chemotherapy in *Leishmania* must target both PTR1 and DHFR-TS (Nare *et al.* 1997a). Significantly, in several studies in which small collections of pteridine analogs were examined, poor correlations of inhibition of parasite growth and DHFR-TS activity were observed (Sirawaraporn *et al.* 1988; Berman *et al.* 1989; Scott *et al.* 1987a). It is possible that the activities of PTR1 or as yet uncharacterized enzymes might account for the lack of correlation.

In this report, we have tested a larger collection of pteridine analogs for activity against purified *Leishmania major* PTR1, in an effort to identify compounds targeting this enzyme specifically. These will provide useful tools for probing the activity of this enzyme *in vitro* and *in vivo.* For a number of compounds showing good inhibitory activity against PTR1, we have determined their activity against *L. major* DHFR-TS and human DHFR, and against wild-type *L. major* promastigotes grown *in vitro.* These studies thus provide information at the biochemical level for rationally establishing potential therapeutic efficacy.

In mammalian cells, the availability of mutant lines exhibiting different mechanisms of antifolate resistance has proven instrumental in testing the action of prospective inhibitors in a more cellular context. We have taken a similar approach here in *Leishmania,* for a subset of compounds showing good activity against PTR1 and wild-type *Leishmania.* In these studies, we used wild-type parasites and mutant cell lines having altered intercellular PTR1 or DHFR-TS levels. Due to the fact that laboratory-selected drug resistant *Leishmania* usually show multiple mechanisms of MTX resistance, we have instead employed lines obtained by targeted deletion of either *PTR1* or *DHFR-TS,* as well as lines exhibiting PTR1 overproduction. These provide an important genetic test of the specificity of targeting of antifolate compounds within the parasite itself, one that in fact yielded some unanticipated findings.

MATERIALS AND METHODS

Enzyme preparations and assays. L. major PTR1 and DHFR-TS were purified from recombinant *Escherichia coli* cells engineered to overproduce these enzymes, as previously described (Nare *et al.* 1997a,b). Human DHFR was prepared using an overproducing bacterial strain (*E. coli* M15/pREP4/pDS5hDHFR) made available to us by Dr. Raymond Blakley. A slight modification of a published procedure (Prendergast *et al.* 1988) was used to purify human DHFR. Methotrexate–agarose (Sigma) was substituted for the methotrexate–Sepharose and the final isoelectric focusing step was omitted. All three enzymes were homogeneous when analyzed electrophoretically on Coomassie-stained denaturing polyacrylamide gels.

The initial velocities of NADPH-dependent reduction of folate or 7,8-dihydrofolate, catalyzed by PTR1 and DHFR, respectively, were measured at 30°C spectrophotometrically as previously described (Nare *et al.* 1997a,b). Standard assays were done at concentrations of folate or dihydrofolate which were 10-fold the K_M. Standard assays of PTR1-catalyzed reactions were made using solutions containing 20 mM potassium phosphate, pH 6, 5 mM dithiothreitol, 0.1 mM NADPH, 36 μM folate, and 1 μM enzyme. Some assays were done at 0.5 and 2 μM PTR1 to test dependence of IC_{50} values on the enzyme concentration. Standard assays of *L. major* DHFR-TS activity were made using solutions containing 50 mM Tes, pH 7.4, 5 mM dithiothreitol, 0.1 mM NADPH, 15 μM dihydrofolate, and 10 nM enzyme. Standard assays of human DHFR activity were done with solutions containing 50 mM Tris, pH 7.0, 5 mM dithiothreitol, 0.1 mM NADPH, 15 μM dihydrofolate, and 80 nM enzyme. All assays were initiated by addition of enzyme. Samples of potential inhibitors dissolved in DMSO were mixed thor-

oughly in the assay solutions prior to introducing an enzyme. With this precaution, the activity of all three enzymes were unaffected by up to 1% DMSO.

For determinations of IC_{50} values, initial velocities were measured at four or more concentrations, showing from <10% to >80% of the control activity. The values of IC_{50} were calculated from the dependence of initial velocities on inhibitor concentrations using the equation

$$v_i/v_o = (1 - C_i/IC_{50})^{-1}, \quad (1)$$

where v_i and v_o are the initial velocities in the presence and absence of inhibitor, and C_i is the concentration of inhibitor. IC_{50} values measured several times independently varied less than 30%.

Inhibition of PTR1 as a function of folate or NADPH concentrations was determined for a few compounds. Initial velocities in the absence and presence of inhibitor (at a concentration equal to its IC_{50}) were measured between 4 and 64 μM NADPH at 36 μM folate and between 2 and 36 μM folate at 100 μM NADPH. Apparent values for V_{max} and K_M were calculated by fitting the data at each fixed concentration of inhibitor and second substrate to the Michaelis–Menten equation using the program KaleidaGraph.

The reversibility of PTR1 inhibition and its dependence upon NADPH was examined as follows, using compounds **25, 31, 36,** and **67.** Solutions were prepared containing 5 μM PTR1 and 6 μM inhibitor, containing 0.1 mM or no NADPH, in 1 mL of buffer (20 mM potassium phosphate, pH 6). Control solutions contained only PTR1 or PTR1 and NADPH. Each sample was concentrated to a volume of *ca.* 0.05 mL using a Centricon-10 unit, diluted by addition of 1 mL of buffer. The concentration and dilution process was done twice more and should have diluted unbound small molecules at least 1000-fold. Each sample was then concentrated again and assayed for protein and PTR1 activity.

The binding affinity of several compounds for the PTR1–NADPH complex was determined directly using a fluorescence quenching method. The fluorescence intensity of solutions of 0.5 μM PTR1 and 0.1 mM NADPH in 20 mM potassium phosphate at pH 6 was measured as a function of inhibitor concentration. The excitation wavelength was 290 or 280 nm, and emission was measured at 450 nm, using a Farrand MK2 fluorescence spectrometer. The fluorescence under these conditions arises from energy transfer between the protein and bound NADPH, which emits at 450 nm. Addition of one of the inhibitors quenched this fluorescence, which plateaued at a lower value at 0.5 μM inhibitor. In some cases, excess inhibitor produced small gradual increases in fluorescence due to fluorescence by the unbound compound. Using the initial and plateau fluorescence intensities and the quenching observed at subsaturating inhibitor concentrations, the fraction of PTR1–NADPH complex bound to inhibitor was calculated. The data were used to calculate values of K_d by a method which accounts for the depletion of free inhibitor due to PTR1 binding (Janin *et al.* 1969).

Candidate inhibitors. Compounds **1, 2, 3, 7, 8, 9, 14, 17, 18, 19, 39,** and **40** were the gifts of Dr. Wolfgang Pfleiderer. Compounds **10, 42, 46, 47, 48,** and **51** were generously provided by Dr. Steven Bailey. Compounds **4, 5, 6, 11, 12, 13, 15, 16, 22, 23, 24, 26, 27, 29–38** inclusive, **41, 44, 45, 49, 72, 73** (2,4-diamino-6-oxo-pteridine), and **74** (7,8-dihydro **73**) were generously provided by Dr. John J. McCormack. Dr. Jill Gready donated samples of compounds **52, 53,** and **54.** Compounds **28** (triamterene), **43, 50, 58** (pyrimethamine), and **60** (trimethoprim) were purchased from Sigma. Samples of compounds **59** and **61–71** inclusive were from the collection of the Drug Synthesis and Chemistry Branch of the National Cancer Institute, and were made available with the aid of Ms. Jill Johnson. All of the above compounds were dissolved in DMSO.

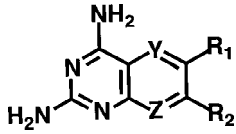
Compounds **20** and **21** (methotrexate and aminopterin, respectively, from Sigma), **55** (5,10-dideaza-5,6,7,8-tetrahydrofolate, a gift from Dr. Chuan J. Shih), and **56** (10-formyl-5,8-dideazafolate, purchased from Dr. John Hynes) were each dissolved in 75 m*M* $NaHCO_3$. Compound **57** (10-methyl-5,8-dideaza-5,6,7,8-tetrahydrofolate, a gift from Dr. M. G. Nair) was prepared in 0.1 *N* NaOH. Compound **25** (O/129) was purchased from Sigma as its phosphate salt and dissolved in water. Solution concentrations of all of the compounds tested in this study were calculated on a weight basis.

Cell lines and culture. All lines of *Leishmania* were derived from *L. major* strain LT252 clone CC-1 and cultured in M199 medium containing 10% fetal bovine serum and various supplements as previously described (Kapler *et al.* 1990). Null mutant *Leishmania* lacking *DHFR-TS* (*dhfr-ts*$^-$) or *PTR1* (*ptr1*$^-$) were created by targeted disruption of both alleles of each gene (Bello *et al.* 1994; Cruz *et al.* 1991). The *ptr1*$^-$ mutant was grown with 7,8-dihydrobiopterin (2–4 μg/μL; Schircks Laboratories, Jona, Switzerland) and the *dhfr-ts*$^-$ mutant was grown in the presence 10 μg/mL thymidine. The line *ptr1*$^-$/+PTR1 represent the *ptr1*$^-$ mutants transfected with the plasmid pX63NEO-PTR1 and overexpresses PTR1 (Bello *et al.* 1994). For growth inhibition, parasites were seeded at 2×10^5 cells/mL with various concentrations of antifolates and measured by counting in a Coulter Counter (Model Zf) at a time when those grown in the absence of drugs were in the late log phase of growth. The EC_{50} values is defined as the concentration of drug required to decrease *Leishmania* growth by 50%.

RESULTS

Preliminary studies of the inhibition of PTR1 were used to choose compounds for further study. IC_{50} values were determined for compounds which showed significant inhibition of PTR1 at 10 μM (Tables I–III). Most of the compounds having IC_{50} values for inhibition of PTR1 of 1 μM or less were characterized by IC_{50} measurements with *L. major* DHFR-TS,

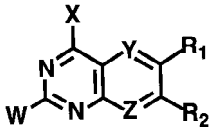
TABLE I
Inhibition of *Leishmania major* PTR1 by 2,4-Diaminopteridines and 2,4-Diaminoquinazolines



Compound	R_1	R_2	PTR1 IC_{50}[a] (μM)
	A. 2,4-Diaminopteridines (Y = Z = N)		
	6-substituted (nonpolar)		
1	$-CH_3$	–H	6
2	$-(CH_2)_2CH_3$	–H	6.5
3	$-CH_2SCH_3$	–H	1.7
4	$-(CH_2)_4CH_3$	–H	0.7
5	$-CH_2CH(CH_3)_2$	–H	4.6
6	$-CH_2OCH_2CH_3$	–H	1.6
7	$-CH_2OCH(CH_3)_2$	–H	5
8	$-CH_2Cl$	–H	1.3
9	$-CH_2Br$	–H	2.1
10	$-C_6H_5$	–H	>5
11	$-C_6H_4$–2–OCH_3	–H	9.3
12	–CH = CH–C_6H_3–3,4–(O_2CH_2)	–H	1.1
13	$-CH_2SC_6H_4$–4–Cl	–H	1.5
	6-substituted (polar)		
14	$-CH_2OH$	–H	2.3
15	$-CH_2CH_2CH(OH)CH_3$	–H	6
16	$-CH_2CH_2COCH_3$	–H	2.3
17	$-CH_2OAc$	–H	2.3
18	$-CH_2OCOCH_2OH$	–H	1.8
19	$-CH_2NHAc$	–H	1.1
20 (MTX)	$-CH_2N(CH_3)C_6H_4CO$–glutamate	–H	1.1
21	$-CH_2NHC_6H_4CO$–glutamate	–H	3.8
	6,7-disubstituted		
22	$-(CH_2)_4-$[b]	–[b]	1.2
23	$-(CH_2)_5-$[b]	–[b]	1.9
24	$-(CH_2)_{10}-$[b]	–[b]	0.5
25	$CH(CH_3)_2$	$-CH(CH_3)_2$	0.5
26	$-SCH_3$	$-CH_3$	0.9
27	$-SCH_3$	$-NH_2$	0.7
28	$-C_6H_5$	$-NH_2$	2.8
	7-substituted		
29	–H	$-CH_2CH(OCH_3)CH_3$	1.6
30	–H	$-CH_2N(Ac)C_6H_3$–3,4–Cl_2	4.8
	B. Quinazolines (Y = Z = CH)		
31	$-OC_6H_4$–4–Cl	–H	0.4
32	$-N(CH_3)C_6H_3$–3,4–Cl_2	–H	0.5
33	$-OC_6H_2$–2,4,5–Cl_3	–H	0.4
34	$-OC_6H_3$–3,4–Cl_2	–H	0.4
35	$-OC_6H_3$–3,5–Cl_2	–H	0.7
36	$-CH_2NHC_6H_3$–3,4–Cl_2	–H	0.9
	C. 5-Deazapteridines (Y = CH, Z = N)		
37	$-(CH_2)_3CH_3$	–H	0.7

[a] Concentration inhibiting PTR1 activity by 50%, determined as described under Materials and Methods.
[b] 6,7-Bridged compound.

TABLE II
Inhibition of *Leishmania major* PTR1 by other Pteridines and Analogs



Compound	W	X	Y	Z	R_1	R_2	PTR1 IC_{50}[a] (μM)
38	$NHCH_3$	NH_2	N	N	$-CH_3$	–H	>10
39	NHAc	NH_2	N	N	$-CH_2OAc$	–H	>10
40	NHAc	NHAc	N	N	$-CH_2OAc$	–H	>10
41	OH	NH_2	N	N	$-C_6H_5$	$-C_6H_5$	>10
42	H	NH_2	N	N	–H	–H	>10
43	H	H	N	N	$-CH_3$	$-CH_3$	>10
44	OH	H	N	N	$-CH_3$	$-CH_3$	>10
45	OH	H	N	N	–H	–H	>10
46	H	OH	N	N	–H	–H	>10
47	H	OH	N	N	–H	$-CH_3$	>10
48	H	OH	N	N	$-CH_3$	$-CH_3$	>10
49	OH	OH	N	N	$-CH_3$	$-CH_3$	>10
50	OH	OH	N	N	–H	–H	>10
51	CH_3	OH	N	N	–H	–H	>10
52	NH_2	OH	CH	NCH_3	–H	$-CH_3$	30
53	NH_2	OH	CH	N	–H	$-CH_3$	70
54	NH_2	OH	N	NCH_3	$-CH_3$	$-CH_3$	30
55	NH_2	OH	CH_2[b]	NH	$-CH_2CH_2C_6H_5CO$–glutamate	–H	>100
56	NH_2	OH	CH	CH	$-CH_2N(CHO)C_6H_4$–CO–glutamate	–H	118
57	NH_2	OH	CH_2[b]	CH_2	$-CH_2N(CH_3)C_6H_4$–CO–glutamate	–H	17

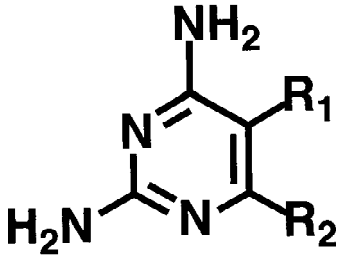
[a] Concentration inhibiting PTR1 activity by 50%, determined as described under Materials and Methods.
[b] 5,6,7,8-Tetrahydro.

human DHFR, and EC_{50} values for inhibition of parasite growth.

The 2,4-diaminopyrimidine structure is a minimal scaffold for PTR1 inhibition. A wide variety of 2,4-diaminopteridines and 2,4-diaminoquinazolines inhibit PTR1 with submicromolar IC_{50}s (Table I). For example, replacement of the 4-oxo group by an amino group in the substrate folate produces the inhibitory compound **21.** Blockage or replacement of the 2-amino or 4-amino groups results in loss of inhibitory potency (Table II). PTR1 is also inhibited by various 2,4-diaminopyrimidines (Table III). The 2,4-diaminopyrimidine scaffold is also common to many effective DHFR inhibitors. Although the inhibitory potency of the compounds listed in Tables I and III against either PTR1 or DHFR-TS depends upon how each compound is substituted, some of the most potent PTR1 inhibitors are very poor DHFR-TS inhibitors (cf. Table IV).

Structure–activity relationships for PTR1 inhibition. The data in Tables I–III provide the first broad perspective into the structural requirements for an effective PTR1 inhibitor beyond the 2,4-diaminopyrimidine scaffold and reveals some general trends. 2,4-Diaminopteridines with either hydrophobic or polar 6-substituents are reasonably good PTR1 inhibitors, but 6,7-disubstituted 2,4-diaminopteridines generally have lower IC_{50}s than do 6-monosubstituted compounds. Direct attachment of a phenyl substituent at the 6 position of the pteridines led to some loss of PTR1 inhibitory potency (Table I, compare compounds **10** and **11** with **12** and **13,** and compare compounds **28** with **26** and **27**). The potent inhibition of PTR1 by the 2,4-diaminoquinazolines demon-

TABLE III
Inhibition of *Leishmania major* PTR1 by 2,4-Diaminopyrimidines



Compound	R_1	R_2	PTR1 IC_{50}[a] (μM)
58	$-C_6H_5$–4–Cl	$-CH_2CH_3$	2.7
59	$-C_6H_4$–3,4–Cl_2	$-CH_2CH_3$	2
60	$-CH_2C_6H_2$–3,4,5–$(OCH)_3)_3$	–H	12
61	$-C_6H_5$	$-CH_2CH_2CONH-C_6H_3$–3,4–$(CH_3)_2$	>300
62	$-C_6H_5$	$-CH_2CH_2CONH-C_6H_4$–4–Cl	>300
63	$-C_6H_5$	$-CONHC_6-H_3$–3,4–Cl_2	300
64	$-C_6H_5$–2–CH_3	–CONH(2–thiazolyl)	46
65	$-C_6H_5$	–CONH(2–thiazolyl)	>300
66	$-(CH_2)_4C_6H_5$–4–NH_2	$-CH_3$	1
67	$-(CH_2)_4C_6H_5$	$-CH_3$	0.7
68	$-(CH_2)_3C_6H_5$	$-CH_3$	1.5
69	$-(CH_2)_4C_6H_5$	–CHO	1.3
70	$-(CH_2)_4C_6H_5$	$-CH_2C_6H_5$	0.4
71	$-(CH_2)_4C_6H_5$	$-CH=CH-C_6H_5$–4–NO_2	0.5
72	$-(CH_2)_4-$[b]	–[b]	1

[a] Concentration inhibiting PTR1 activity by 50%, determined as described under Materials and Methods.

[b] 5,6-Bridged compound.

strates that neither nitrogen 5 nor nitrogen 8 are needed for effective inhibition of PTR1 (cf. compounds **31–37,** Table I). In contrast to the 2-4-diaminoquinazolines, 2-amino-4-oxoquinazolines (compounds **55–57,** Table II) are very weak PTR1 inhibitors, although these compounds effectively inhibit some other folate-dependent enzymes (Baldwin *et al.* 1991; Hynes *et al.* 1997; Scanlon *et al.* 1979).

2-4-Diamino-6-oxopteridine (compound **73**) is itself a very poor PTR1 inhibitor, but its 7,8-dihydro form **(74)** is a potent inhibitor. Compounds **73** and **74** have IC_{50} values of 14 and 0.6 μM, respectively, for PTR1 inhibition. Similarly, the dihydro forms of PTR1 substrates are inhibitory toward PTR1 (Nare *et al.* 1997a), whereas fully oxidized substrates and the fully reduced (5,6,7,8-tetrahydro) pteridine products are not. The potency of compound **74** suggests that the geometry at position 6 is more important for PTR1 inhibition than the geometry and protonation of nitrogen 5. The low IC_{50} value for the 5-deaza-2,4-diaminopteridine **37** is consistent with this suggestion. Incorporation of the 7,8-saturated feature into the structures of other compounds may provide additional potency for PTR1 inhibition.

2,4-Diaminopyrimidines with 5-phenylpropyl or 5-phenylbutyl substituents (Table III, compounds **66–71**) had lower IC_{50}s for PTR1 inhibition than pyrimidines (**58,** pyrimethamine, or **60,** trimethoprim) which lacked the 5-alkyl tether. The former were among the most potent PTR1 inhibitors identified in this study.

The nature of the PTR1–inhibitor interactions. The IC_{50} values for PTR1 inhibition ob-

served with some compounds were similar to the concentration of PTR1 in standard assays (1 μM), and the lowest IC_{50}s seen were about half that value. A substantial fraction of a high-affinity inhibitor, at all concentrations below that of the enzyme, becomes enzyme bound, and the effective limit for any IC_{50} value is one-half the enzyme concentration (Cha 1975). This was demonstrated with PTR1 and compound **31,** for which the IC_{50} increased linearly over a fourfold range of PTR1 concentration. The low turnover number of PTR1 required a much higher PTR1 concentration in the standard assays (1 μM) than we used in the standard DHFR-TS or human DHFR assays (10 and 80 nM, respectively), so the minimal IC_{50} values observable for PTR1 are higher than those for DHFR-TS and DHFR.

A fluorescence assay was used to demonstrate that the affinities for several compounds for PTR1 were in fact substantially greater than suggested by their IC_{50} values. The values of K_d for binding of compounds **4, 31, 36, 37,** and **71** to the PTR1-NADPH complex were 40, 6, 19, 13, and 27 nM, respectively. No interaction between PTR1 and these or other compounds could be detected by fluorescence in the absence of NADPH, supporting the notion that NADPH binding must precede the binding of pteridine analogues. Kinetic studies of catalysis by PTR1 (unpublished data) indicate that NADPH must bind to PTR1 before the enzyme is competent to bind a pteridine substrate; similar kinetic behavior of other short-chain dehydrogenases/reductases (e.g., dihydropteridine reductase) has been described (Kiefer *et al.* 1996).

Inhibition of PTR1 by both a 2,4-diaminopteridine (compound **25**) and a 2,4-diaminopyrimidine (compound **67**) was noncompetitive versus either folate or NADPH (data not shown), consistent with slowly reversible, tight binding inhibition by these compounds. This idea is consistent with the observation that inhibition of PTR1 by compounds **25, 31, 36,** and **67** was only partially reversed by cycles of dilution and concentration sufficient to dilute unbound small molecules by >1000-fold. After this treatment, PTR1 was still inhibited by 52 to 77%. The persistent inhibition required the presence of NADPH, since PTR1 samples treated identically in the presence of inhibitors but lacking NADPH, or with NADPH alone, had the same specific activity as untreated enzyme. This indicates that slowly reversible, tight binding of these pteridine analogs occurs only after NADPH binds.

Correlations between PTR1 and DHFR-TS inhibition and antileishmanial activity. For a number of compounds showing good activity against PTR1, we determined IC_{50}s against the *L. major* DHFR-TS and human DHFR (Table IV). This provided a biochemical test of specificity, in that compounds targeting the two *Leishmania* enzymes but not human DHFR would seem logical drug candidates. Some agents showed good inhibitory activity against all three enzymes (such as compounds **24, 31, 32, 33, 34,** and **71**), whereas another group showed good activity against PTR1 but not DHFR-TS or DHFR (compounds **12, 19, 26, 69, 72,** and **74**). Compounds such as **21** (aminopterin), **56,** and **57,** known to potently inhibit DHFR but having little activity against PTR1, are excluded from Table IV since this portion of our study only included those compounds with IC_{50} values of ca. 1 μM or lower against PTR1. Inhibitors showing preferential activity against DHFR-TS or PTR1 will be useful pharmacological tools for probing the role of these two enzymes in the future.

Those PTR1 inhibitors which did not also inhibit DHFR-TS showed little antileishmanial activity (Table IV). Several of those compounds which had good activity in inhibiting *Leishmania* growth had lower IC_{50}s for *L. major* DHFR-TS than for human DHFR. Most of these were quinazolines (compounds **31, 34,** and **35**) with the notable exception of compound **70,** a 2,4-diaminopyrimidine. These are potential leads for the development of useful human therapeutic agents.

Genetic tests of inhibitor specificity. For 10 compounds (MTX and 9 other compounds with good activity against wild-type *Leishmania*), we tested the ability to inhibit the growth of engi-

TABLE IV
Inhibition of *Leishmania major* DHFR-TS and Human DHFR Activities and of the Growth of *Leishmania major* Promastigotes *in Vitro*[a]

Compound	IC_{50} (μM) for[b]			EC_{50} (μM) for[c]			
	PTR1	*L. major* DHFR-TS	Human DHFR	Wild type	*ptr1*$^-$	*ptr1*$^-$/+PTR1	*dhfr-ts*$^-$
Active against *Leishmania*							
Pteridines and quinazolines							
20 (MTX)	1.1	0.005	0.04	0.3 ± 0.1	0.03 ± 0.02	20 ± 5	>100
24	0.5	1.5	0.6	1.0 ± 0.2	0.3 ± 0.1	2 ± 0.4	1.0 ± 0.3
31	0.4	0.14	2.1	0.5 ± 0.1	0.02 ± 0.01	1.3 ± 0.1	0.9 ± 0.1
32	0.5	0.46	0.36	0.10 ± 0.03	0.013 ± 0.004	0.53 ± 0.07	2 ± 1
34	0.4	0.16	1.4	0.4 ± 0.3	0.3 ± 0.2	0.5 ± 0.2	0.4 ± 0.1
35	0.7	0.34	2.4	0.2 ± 0.1	0.07 ± 0.03	0.3 ± 0.1	0.13 ± 0.08
36	0.9	0.025	0.04	0.6 ± 0.4	0.3 ± 0.2	1.4 ± 0.2	2.0 ± 0.1
Pyrimidines							
67	0.7	2.1	1.5	0.2 ± 0.1	0.012 ± 0.005	0.63 ± 0.03	0.4 ± 0.3
68	1.5	1.3	2.2	0.54 ± 0.02	0.3 ± 0.1	1.5 ± 0.4	0.9 ± 0.2
70	0.4	0.17	7	0.3 ± 0.2	0.09 ± 0.06	0.5 ± 0.1	0.4 ± 0.3
Weak or no activity against *Leishmania*							
Pteridines and quinazolines							
4	0.7	6.4	4	>10	[d]		
12	1.1	>10	>100	>10			
19	1.1	>100	>100	>10			
26	0.9	16	>100	>10			
27	0.7	2.2	7	7.3			
33	0.4	0.16	0.5	1.3			
37	0.7	1.6	3.4	7.3			
74	0.6	>100	>100	>10			
Pyrimidines							
69	1.3	16.5	>10	>5			
71	0.5	0.36	0.25	1.3			
72	1	>50	35	>10			

[a] Compounds are listed in numerical order in the indicated functional and structural groupings.
[b] Errors in the IC_{50} values were 25% or less. The IC_{50} values for PTR1 from Tables I–III are also shown for the sake of comparisons.
[c] The indicated uncertainties in the EC_{50} values are standard errors of two to five independent experiments.
[d] Blank entries indicate values which were not determined.

neered *L. major* mutants with known alterations in pteridine metabolic enzymes (Tables IV and V). Of these, *ptr1*$^-$ mutants are known to exhibit hypersensitivity to methotrexate, while PTR1-overexpressing lines are highly resistant to MTX (Bello *et al.* 1994; Papadopoulou *et al.* 1994). It should be noted that in order to maintain viability, *ptr1*$^-$ parasites must be cultivated in the presence of reduced biopterin (Bello *et al.* 1994), and thus inhibition tests of this parasite cannot discern effects relating to the inhibition of the pterin (as opposed to folate) reductase pathway. The *dhfr-ts*$^-$ parasites, which must necessarily be cultivated in the presence of thymidine, show high resistance to MTX (Gueiros-Filho and Beverley 1996). This resistance probably can be attributed to the absence of thymidylate synthase, as this is the only known cellular enzyme capable of oxidizing reduced folates and its absence thus can spare reduced folate pools. We did not test lines deficient in folate uptake, as unlike *L. donovani* (Kaur *et al.*

TABLE V
Three Groups of Compounds are Apparent from the Patterns of Apparent Sensitivity or Resistance to Growth Inhibition Evoked by Altered Intracellular Levels of *Leishmania major* PTR1 or DHFR-TS

	IC_{50} (μM)		Ratio of indicated EC_{50} to wild-type EC_{50}[a]		
	L. Major			*ptr1*$^-$/	
Compound	PTR1	DHFR-TS	*ptr1*$^-$	+PTR1	*dhfr-ts*$^-$
MTX-like compounds					
20 (MTX)	1.1	0.005	0.1	67	>333
32	0.5	0.46	0.1	5	20
PTR1-sensitive compounds					
31	0.4	0.14	0.04	3	2
67	0.7	2.1	0.06	3	2
DHFR-TS, PTR1-insensitive compounds					
24	0.5	1.5	0.3	2	1
34	0.4	0.16	0.8	1	1
35	0.7	0.34	0.4	1	0.7
36	0.9	0.025	0.5	2	3
68	1.5	1.3	0.6	3	2
70	0.4	0.17	0.3	1.5	1

[a] The EC_{50} values used to calculate the ratios are those shown in Table IV.

1988), those available in *L. major* show very minor reductions in uptake (Ellenberger and Beverley 1987a,b). From the structural features of these compounds, or studies in other organisms, it appears likely that the great majority of compounds listed in Tables I–III (other than conjugated pterin derivatives) would be permeable to the cell.

Of the nine compounds tested in addition to MTX **(20),** one **(32)** resembled MTX in the pattern of resistance and sensitivity exhibited by the four lines tested. This is most conveniently summarized (Table V) by the ratios of the EC_{50}s of the most sensitive (*ptr1*$^-$), PTR1-overproducing and most resistant (*dhfr-ts*$^-$) lines to the EC_{50} of the wild type. These ratios ranged from 0.04 to >333. Remarkably, none of the other compounds tested showed a MTX-like pattern. Six of them exhibited a fairly uniform inhibition profile, showing no more than seven-fold variation among the indicator lines (compounds **24, 34–36, 68,** and **70**). Two showed hypersensitivity toward the *ptr1*$^-$ line (compounds **31** and **67**), while remaining fairly uniform against the *dhfr-ts*$^-$ and PTR1-overexpressing lines.

Notably, the grouping of the compounds by their activities against the indicator cell lines does not show any relation to their relative activities against PTR1 or DHFR-TS. For example, the MTX-like compound **32** has roughly similar enzymatic IC_{50}s as those of compounds **34** and **70** which were indifferent to the variation of PTR1 or DHFR-TS levels. It is remarkable that PTR1 overexpression, which leads to a high level of pteridine reductase activity (200-fold higher than in wild-type cells) and methotrexate resistance in a variety of circumstances (Bello *et al.* 1994; Nare and Beverley, manuscript in preparation), confers very little resistance to compounds **24, 34–36, 68,** and **70.** This group includes pteridines, quinazolines, and pyrimidines. The simplest explanation for these findings is that while these compounds have good activity against the two *Leishmania* pteridine reductases we tested, inside the parasite cell these reductases are not the only significant targets.

DISCUSSION

We identified a set of potent inhibitors of *L. major* PTR1 from a collection of pteridines, pteridine analogs, and pyrimidines. This enzyme, a new member of the short-chain dehydrogenase/reductase family (Callahan and Beverley 1992; Papadopoulou *et al.* 1992; unpublished results), catalyzes the same reactions as DHFR but with a modified substrate specificity (Bello *et al.* 1994; Nare *et al.* 1997a). Although PTR1 and DHFR are unrelated at the primary sequence level (and probably structurally as well), both enzymes are inhibited by compounds containing the 2,4-diaminopyrimidine scaffold. Nonetheless, some of the most potent PTR1 inhibitors identified are impotent against either *L. major* DHFR-TS or human DHFR. In addition to providing an initial structure–activity relationship for PTR1 inhibitors, testing of the compounds in a set of wild type and defined mutant strains of *Leishmania* provided insight into their *in vivo* targets. Several signifi-

cant conclusions resulted: (1) potent inhibition of PTR1 alone is insufficient for growth inhibition, (2) depletion of intracellular PTR1 levels sensitizes the parasites to growth inhibition by some compounds but not all, and (3) several of the compounds studied are likely to act against targets other than or in addition to PTR1 and DHFR-TS.

Our results provide a possible explanation for the disagreement among previous reports regarding the correlation between the inhibition of the DHFR activity of *Leishmania* DHFR-TS by antifolates and the inhibition of parasite growth *in vitro.* One study of 2,4-diaminopyrimidines found a modest correlation between DHFR inhibitory potency and the EC_{50} for inhibition of amastigote proliferation in mouse macrophages (Sirawaraporn *et al.* 1988). In contrast, other studies found little correlation between the ability of some quinazolines (Berman *et al.* 1989) or other 2,4-diaminopyrimidines (Scott *et al.* 1987a) to inhibit *Leishmania* DHFR activity and their potency in inhibiting cell growth. The data for compounds **31, 32,** and **67** in Table IV support the idea that the alternative dihydrofolate reductase activity provided by PTR1 may provide resistance to some compounds which might be expected to inhibit parasite growth on the basis of activity against DHFR-TS.

A surprising finding from our study was that many of the inhibitors with good activity against PTR1 and DHFR-TS enzymes showed no correlation of parasite growth inhibition with the level of expression of either PTR1 or DHFR-TS (Table V). Only compound **32** showed the patterns of sensitivity and resistance expected for an agent like MTX (compound **20**) which primarily targets DHFR-TS and PTR1 (Table V). Two compounds (**31** and **67**) had increased potency against cells which lacked PTR1 activity, but their antileishmanial activity did not depend on the presence of DHFR-TS. For these two, the presence of PTR1 appears to provide a metabolic bypass, but unlike MTX, one that is independent of DHFR-TS. The most surprising result is that for six of the compounds (**24, 34–36, 68,** and **70**), there was essentially no modulation of potency by varying PTR1 or DHFR-TS levels *in vivo* (Table V). The simplest explanation for this result is that these inhibitors may be acting against additional targets in *Leishmania,* beyond PTR1 and DHFR-TS.

The identity(ies) of the prospective new drug target(s) is/are unknown at present. Conceivably, it may not even be a pteridine metabolic enzyme. We consider it more likely that another pteridine metabolic enzyme is the target, given that the active compounds exhibit a spectrum of molecular scaffolds and retain good potency against both PTR1 and DHFR-TS. Cross-inhibition of pteridine metabolic enzymes by antifolates is often observed, as exemplified by the data in Table IV as well as studies of mammalian pteridine metabolic enzymes (Shih *et al.* 1997). *Leishmania* are purine auxotrophs and thus lack several important folate-dependent enzymes of this pathway. However, there are several known folate-requiring enzymes in *Leishmania* which are prospective targets (Scott *et al.* 1987b). One such candidate is serine hydroxymethyltransferase, which catalyzes the conversion of L-serine and 5,6,7,8-tetrahydrofolate to glycine and 5,10-methylene-5,6,7,8-tetrahydrofolate, and is hence required for the biosynthesis of thymidylate. Another target candidate is 5,10-methylene-5,6,7,8-tetrahydrofolate reductase, which generates 5-methyl-5,6,7,8-tetrahydrofolate and is therefore required for the conversion of L-homocysteine to L-methionine. Future studies, both biochemical and genetic, will be required to determine the mode of action of compounds **24, 34–36, 68,** and **70** against *Leishmania.*

In conclusion, our studies have provided biochemical support for strategies targeting the DHFR-TS/PTR1 pteridine reductase pathway and identification of a class of inhibitors effective primarily against PTR1. In studies to be reported elsewhere, we show that compound **25** (O/129), which resembles MTX in its enzymatic and cellular inhibition profiles, is capable of specifically inhibiting *L. major* amastigote growth in cultured macrophages *in vitro* (Nare and Beverley, manuscript in preparation). Moreover, our studies have revealed a new class

of antifolate compounds with activity against a prospective new *Leishmania* drug target. Thus, by combining biochemical and genetic tests of potential antifolates against purified enzymes and mutants bearing specific alterations in pteridine metabolism, we have been able to test rigorously the *in vivo* contributions of individual targets to drug sensitivity and resistance. This approach will be a powerful tool in the search for drugs and new drug targets in *Leishmania.*

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