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PTR1*-dependent synthesis of tetrahydrobiopterin contributes to oxidant susceptibility in the trypanosomatid protozoan parasite *Leishmania major

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Abstract

Leishmania must survive oxidative stress, but lack many classical antioxidant enzymes and rely heavily on trypanothione-dependent pathways. We used forward genetic screens to recover loci mediating oxidant resistance via overexpression in *Leishmania major*, which identified pteridine reductase 1 (PTR1). Comparisons of isogenic lines showed *ptr1*⁻ null mutants were 18-fold more sensitive to H₂O₂ than PTR1-overproducing lines, and significant 3-5 fold differences were seen with a broad panel of oxidant-inducing agents. The toxicities of simple nitric oxide generators and other drug classes (except antifolates) were unaffected by PTR1 levels. H₂O₂ susceptibility could be modulated by exogenous biopterin but not folate, in a PTR1-but not dihydrofolate reductase-dependent manner, implicating H₄B metabolism specifically. Neither H₂O₂ consumption, nor the level of intracellular oxidative stress, was affected by PTR1 levels. Coupled with the fact that reduced pteridines are at least 100-fold less abundant than cellular thiols, these data argue strongly that reduced pteridines act through a mechanism other than scavenging. The ability of unconjugated pteridines to counter oxidative stress has implications to infectivity and response to chemotherapy. Since the intracellular pteridine levels of *Leishmania* can be readily manipulated, these organisms offer a powerful setting for the dissection of pteridine-dependent oxidant susceptibility in higher eukaryotes.

INTRODUCTION

Trypanosomatid protozoan parasites of the genus *Leishmania* include a number of species causing diseases affecting more than 12 million people worldwide (Desjeux 2004). These parasites multiply as two forms: flagellated extracellular promastigotes that reside within the digestive system of the sand fly vector, and as non-flagellated intracellular amastigotes living in the phagolysosome of the vertebrate macrophage. Phagocytosis of *Leishmania* by macrophages can stimulate a respiratory burst, generating reactive oxygen species (ROS) including O₂⁻ and H₂O₂, and parasites must suppress, survive, or recover from this oxidative attack (Denkers and Butcher 2005). The induction of oxidant stress is an important action of clinical pentavalent antimonials, and antioxidant systems contribute to antimonial resistance (Ashutosh, et al. 2007, Wyllie, et al. 2004).

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In dealing with oxidative stresses organisms have two basic options: ‘upstream’ methods involving direct detoxification of the various reactive oxidant species (ROS), or ‘downstream’ pathways repairing oxidatively damaged molecules (Davies 1995). The balance between and the consequences of disruptions in upstream vs. downstream oxidant susceptibility pathways varies amongst organisms (Fang 2004). Notably, *Leishmania* lack many classical systems that detoxify H₂O₂ in other organisms, such as catalase and the selenoprotein glutathione peroxidase (Krauth-Siegel, et al. 2007). Instead, their antioxidant metabolism relies on the thiol N₁,N₈-bis(glutathionyl)spermidine (trypanothione, T[SH]₂), and a host of antioxidant systems that depend on reducing equivalents passed to T[SH]₂ by trypanothione reductase (Fairlamb and Cerami 1992). These include downstream redox intermediates, such as ascorbate, tryparedoxin and ovoidiol, (Ariyanayagam and Fairlamb 2001, Jaeger and Flohe 2006, Krauth-Siegel and Ludemann 1996) and antioxidant enzymes such as ascorbate peroxidase, trypanothione *S*-transferase, peroxiredoxins, and cysteine homologs of classical glutathione peroxidase (Krauth-Siegel, et al. 2007). In contrast to these ‘upstream’ pathways, ‘downstream’ pathways have received less attention in parasites.

Another set of redox-active metabolites in *Leishmania* are the unconjugated pteridines, whose roles in oxidative stress are less well understood. For example, while many studies have shown or assumed that reduced pteridines such as tetrahydrobiopterin (H₄B; the most common eukaryotic pteridine) can scavenge oxidants, others show that H₄B may instead promote oxidative stress through the Fenton reaction (Kirsch, et al. 2003). For pteridines as a group, the biological situation is complex and highly dependent on the specific circumstances, as non-reduced pteridines can act to promote oxidative damage, and pteridines can act as both substrates and inhibitors of enzymes that both generate and protect from oxidants, such as the nitric oxide synthases (reviewed in refs. Oetl and Reibnegger 2002, Werner-Felmayer, et al. 2002). This complexity has led some to conclude that ‘it is impossible to unequivocally predict a physiological net effect of pterins of different oxidation states...in real biological systems’ (Oetl and Reibnegger 2002). One factor that may have contributed to these paradoxical findings is the manner of testing, typically involving simultaneous external application of both pteridines and/or oxidative stresses. This leads to difficulty in distinguishing between the intracellular and extracellular effects of the pteridines. Here we use the unparalleled ability to manipulate pteridine metabolism internally in *Leishmania* to further our understanding of how pteridines can modulate *in vivo* oxidant susceptibility.

Leishmania are pteridine auxotrophs, and use an array of transporters and reductases to acquire and generate reduced pteridines (reviewed in refs. Nare, et al. 1997b, Ouellette, et al. 2002). While dihydrofolate reductases (DHFRs) often can reduce both folates and unconjugated pteridines, *Leishmania* DHFR can only reduce folates (Nare, et al. 1997a). Thus *Leishmania* and other trypanosomatids express a novel pteridine reductase (pteridine reductase 1, PTR1), which is broadly active and can reduce a variety of unconjugated pteridines, as well as folates (Bello, et al. 1994, Nare, et al. 1997a, Wang, et al. 1997). Being relatively insensitive to classic antifolates such as methotrexate, PTR1 acts as a metabolic ‘bypass’ of DHFR inhibition, accounting for the relative inactivity of antifolates against trypanosomatids (Bello, et al. 1994). The known functions of pteridines in mammalian cells include hydroxylation of aromatic amino acids, lipid cleavages and nitric oxide biosynthesis (Kaufman 1963, Kosar-Hashemi and Armarego 1993, Tayeh and Marletta 1989). However, only an NADPH-dependent ether lipid cleavage activity has been described in *Leishmania* (Ma, et al. 1996), the *L. major* genome lacks an identifiable nitric oxide synthase, despite reports of NO synthesis (Wanasen and Soong 2008), and while possessing a phenylalanine hydroxylase, null mutants lacking this enzyme have normal growth, differentiation and virulence (L-F. Lye and SMB, in preparation). Thus the precise role(s) of H₄B in *Leishmania* metabolism remains uncertain.

We and others have previously used genetic selections for drug resistance to identify loci active when overexpressed, either naturally by gene amplification (Beverley 1991, Ouellette, et al. 2004), or engineered after transfection with random gene segments borne on multicopy episomal cosmid vectors (reviewed in Beverley, 2003; Clos and Choudhury 2006). Here we applied this method to the discovery of novel oxidant resistance genes, using treatment with H₂O₂ as the selective pressure. This screen identified *PTR1* as a mediator of susceptibility to oxidative stress, a finding corroborated by studies of isogenic lines lacking or overexpressing *PTR1*. Further studies point to a novel mode of oxidant resistance dependent upon reduced unconjugated pteridines, distinct from the T[SH]₂-dependent antioxidants cited earlier. We advanced and tested several potential mechanisms through which *PTR1*-derived pteridines could modulate oxidant susceptibility. Lastly, we discuss the implications of our findings on parasite survival, virulence and chemotherapy.

MATERIALS AND METHODS

Chemicals and Reagents

Reagents were obtained as follows: biopterin and H₂B (Schircks Laboratories, Jona, Switzerland); H₄B (RBI); methotrexate, thymidine, sodium nitrite, allopurinol, N-methyl-N'-nitro-N-nitrosoguanidine, glutathione, folate, dihydro- and tetrahydrofolate, hydrogen peroxide, bovine catalase, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), pentamidine, (Sigma); 2'7'-dichlorofluorescein diacetate (Molecular Probes); S-nitroso-acetyl-penicillamine; 3-morpholino-sydnominine; S-nitrosoglutathione (Alexis Corporation); paraquat (Aldrich); horseradish peroxidase (Boehringer Mannheim); bovine serum albumin (US Biochemicals); terbinafine (Sandoz Research Institute).

Cell lines and culture conditions

The *L. major* strains LT252 (MHOM/IR/83/IR) clone CC-1, Friedlin (MHOM/IL/81/Friedlin) clones FA1 (avirulent) and FV1 (virulent), were routinely propagated at 26° C as promastigotes in M199 containing 10% (v/v) heat-inactivated fetal bovine serum, adenine (100 µM), hemin (5 µg/ml), and biopterin (2 µg/ml) (Kapler, et al. 1990). Transfection of *Leishmania* by electroporation was performed as described (Kapler, et al. 1990, Ryan, et al. 1993). fdM199 medium is standard M199 lacking folate and thymidine. FDLS-M199 medium is standard M199 lacking folate and thymidine, supplemented with 0.66% (w/v) bovine serum albumin, 1% (v/v) fetal bovine serum and 10 ng/ml H₂ B. *L. major* CC-1 null mutants lacking DHFR-TS (*dhfr-ts*⁻) or *PTR1* (*ptr1*⁻) by targeted gene replacement of both alleles were described previously (Bello, et al. 1994, Cruz, et al. 1991), and maintained in media supplemented with 10 µg/ml thymidine or 2-4 µg/ml reduced biopterin respectively. The CC-1 lines *ptr1*⁻/*+PTR1* and *dhfr-ts*⁻/*+DHFR-TS* represent the respective null-mutants transfected with episomal expression plasmids pX63NEO-*PTR1* (Bello, et al. 1994) or pK300 (Kapler, et al. 1990), and overexpress *PTR1* and DHFR-TS respectively. Similarly, the FV1 *ptr1*⁻/*SSU::PTR1* overexpressor is a chromosomal null *ptr1*⁻ mutant complemented with a copy of the *PTR1* gene integrated in the small subunit ribosomal locus, which also confers high level overexpression (D. Scott *et al.* manuscript in preparation).

Multi-copy selection for hydrogen peroxide resistance

We previously described the construction of cosmid libraries in the vector cLHYG containing ~30 kb inserts of *L. major* strain FV1 DNA, generated by either partial digestion with *Sau3A* or random shear, and their mass transfection into the *L. major* strain FA1 (Cotrim, et al. 1999, Ryan, et al. 1993). Strain FA1 and the cosmid library transfected FA1 pool were inoculated into M199 media containing 600 µM H₂O₂ at a final concentration of 1 × 10⁶ parasites/ml, and allowed to incubate at 26°C for several weeks.

Characterization of the Gene Responsible for H₂O₂ Resistance

Insertional mutagenesis was performed using a modified Ty1-based *in vitro* transposition system (Devine and Boeke 1994, Garraway, et al. 1997). Transposition reactions were carried out using 2 µg of cHP DNA as target, using conditions previously described (Garraway, et al. 1997). A set of single insertions was obtained and the general location of the TyK artificial transposon was mapped by enzymatic digestion with *Hind*III. TyK insertions were further analyzed by polymerase chain reaction with the following primers: SMB-392 (5'-cgcgatcccatATGACTGCTCCGACC) and SMB-393 (5'-ggcggatccTCAGGCCCGGGTAAGGCTGTA).

Determination of *Leishmania* survival

For growth inhibition, log phase promastigotes (2×10^5 cells/ml) were incubated with H₂O₂ or other test drugs in 10 ml FDLS-M199. *Leishmania* were irradiated (0-250 mJ/cm²) in the GS Gene Linker UV chamber (Biorad) and allowed to recover in the presence or absence of visible light. Cell numbers were estimated with a Coulter Counter (Model Zf). The EC₅₀ is defined as the condition decreasing growth by 50%, measured at a time when controls had reached late log phase ($< (1 \times 10^7/\text{ml})$).

Measurement of H₂O₂ removal by *Leishmania*

Log phase *Leishmania* were harvested and resuspended in a buffer containing 5 mM KCl, 80 mM NaCl, 2 mM MgCl₂, 16.2 mM Na₂HPO₄, 3.8 mM NaH₂PO₄, 50 mM D-glucose, 100 µg/ml phenol red, pH 7.5 and 20 µM H₂O₂. 1 ml aliquots were removed periodically and H₂O₂ levels determined by measuring absorbance at 610 nm as described (Penketh 1986, Pick and Keisari 1980). The concentration of H₂O₂ stocks was determined spectrophotometrically at 230 nm assuming an extinction coefficient of 81/M/cm at 230 nm (Homan-Muller, et al. 1975).

Measurement of intracellular oxidative stress

Logarithmic phase *Leishmania* were maintained in fdM199 media without biopterin for 24 h to deplete the internal pteridine pools, harvested, washed and resuspended (1×10^7 cells/ml) in Hanks balanced salt solution without phenol red, and loaded with 10 µM DCFH-DA for 15 min. Aliquots (1 ml) were removed at various time points, centrifuged and DCF fluorescence was measured spectrofluorimetrically (excitation 485 nm; emission 530 nm) (Keston and Brandt 1965). To test the involvement of peroxides on DCF-DA oxidation, catalase was added (5000 U/ml) for 5 min and washed off the cells prior to loading with DCF-DA.

Determination of parasite viability by MTT assay

Parasites were harvested from mid-log cultures and resuspended at 8×10^6 /ml in M199 media containing 2 µg/ml biopterin. H₂O₂ was then added and the cells incubated at 26°C for 2 h. Triplicate 1 ml samples were removed, the cells harvested by centrifugation and then resuspended in 1 ml fresh M199 containing 400 µg/ml MTT. These samples were incubated for a further 2 h at 26°C and the cells pelleted again. The cell pellets were lysed and the formazan product dissolved by addition of 2 % (w/v) sodium dodecyl sulphate in DMSO. The reduced product was quantified by measuring its absorbance at 570 nm versus a media blank. Standard curves showed that this assay gave a linear response from samples containing 1×10^6 to 8×10^6 viable parasites.

RESULTS

Genetic screening for novel H₂O₂ resistance genes

Previously we generated a library of 18,000 independent *L. major* transfectants, each bearing a ~30 kb segment of WT genomic DNA inserted in the multicopy episomal shuttle vector cLHYG (Cotrim, et al. 1999). Cultures of the WT parent and the pooled transfectant library were propagated in the presence of 600 μ M H₂O₂ for several weeks. While selections of untransfected WT parasites did not yield survivors, parasites grew out from the H₂O₂ treated transfectant library. This population showed a 10-fold increase in its EC₅₀ for H₂O₂, compared to the parent line (not shown). DNA was recovered from the H₂O₂-resistant population and transformed into *E. coli*, and analysis of 24 colonies showed that all contained independently the same cosmid, which was termed cHP. Re-transfection of the WT line with cHP conferred up to 7-fold resistance to H₂O₂ (Fig. 1B), confirming its activity.

Molecular analysis of cHP showed it contained a segment of the *L. major* genome termed the H-region (Beverley, et al. 1984; Fig. 1B), a locus frequently amplified in lines selected for resistance to a variety of drugs (Beverley 1991, Ouellette, et al. 2004). The H region contains at least 14 genes, most of whose functions are unknown, and includes *PTR1*, *PGPA* and *HTFB*, which mediate resistance to antifolates, arsenicals/antimonials, and the sterol synthetic inhibitor terbinafine, respectively. To identify the H₂O₂ resistance gene(s), we used insertional mutagenesis with a TyK artificial transposon to generate a library of independent insertions, which were mapped and tested following transfection into WT *L. major* (Fig. 1B). While most insertions had no effect, insertions disrupting *PTR1* (cHP-PTR1::TyK) abrogated H₂O₂ resistance (Fig. 1B). Consistent with the established role of *PTR1* in antifolate resistance, cHP transfectants were methotrexate resistant, while cHP-PTR1::TyK transfectants had sensitivities similar to controls (data not shown). These data identified *PTR1* specifically as the sole cHP oxidant resistance gene.

PTR1 Protects Against Oxidative Stress

To probe the role of PTR1 in oxidant susceptibility, we employed isogenic lines in the *L. major* CC-1 strain, where *PTR1* had been specifically deleted by homologous gene replacement (*ptr1*⁻), or overproduced using an episomal expression vector (*ptr1*⁻/+*PTR1*; (Bello, et al. 1994, Nare, et al. 1997a). Focusing first on agents known to generate oxidative stress, the *ptr1*⁻ line was nearly 4-fold more sensitive to H₂O₂, glucose oxidase (which generates H₂O₂ in the presence of glucose), and primaquine, a redox-cycling drug (which produces O₂⁻ and H₂O₂) (Table 1; *p* < 0.01;). Modest sensitivity was seen to a second redox-cycling agent, paraquat (1.3-fold), although this was not statistically significant, and no change was seen in susceptibility to *tert*-butylhydroperoxide, which forms alkoxyl, peroxy and hydroxyl radicals (.OH) (Table 1). Conversely, overexpression of PTR1 led to significant increases in oxidant resistance with all agents tested, ranging from 1.5 fold for primaquine to 4.8 fold for H₂O₂ (Table 1, Fig. S2). A small effect was seen for glucose oxidase/glucose stress, although this was not statistically significant (Table 1).

We found in these studies that the ratio of the EC₅₀ of the PTR1 overexpressor to that of the *ptr1*⁻ mutant provided a convenient parameter to assess the impact of PTR1 on oxidant susceptibility. Thus the differential sensitivity between the PTR1-overproducer and the *ptr1*⁻ null mutant was 18-fold with H₂O₂, and ranged from 3- to 5-fold with all other oxidant generating systems, with strong statistical significance for all (Table 1). These data revealed a general role for PTR1 in determining the susceptibility of *Leishmania* to oxidative stress.

PTR1 does not affect resistance to nitrosative stress or other agents

Nitric oxide (NO) is an important microbicidal product of macrophages and is cytotoxic to *Leishmania in vitro* (Liew, et al. 1990), so we tested whether *PTR1* also mediated resistance to reactive nitrogen species (RNS). All three lines displayed equivalent sensitivity to the NO-generating compounds sodium nitrite and S-nitrosoglutathione (Table 1), suggesting that *PTR1* was not involved in protection against nitrosative stress. S-nitrosopenicillamine showed opposite effects to that seen with oxidants, with *ptr1*⁻ showing slight resistance and the *PTR1* overexpressor showing sensitivity; however the magnitude of these effects were small and not significant (~1.2 fold; Table 1). An apparent exception was the finding that the *ptr1*⁻ line was 5.3-fold more sensitive than the *PTR1* overproducer to morpholinosydnonimine (SIN-1), a generator of both NO and O₂⁻. This could suggest a role of PTR1 in protection against peroxynitrite, a product of the reaction between NO and O₂⁻, however an alternative explanation is that resistance arose solely to the O₂⁻ formed by SIN-1.

Tests of drugs not thought to act through oxidative stress such as allopurinol, terbinafine or pentamidine showed no significant differences between *ptr1*⁻, WT and *PTR1* overexpressing lines (Table 1). The genomes of *Leishmania major* and other trypanosomatids encode numerous DNA repair pathway activities, including DNA photolyase (LmjF33.0470) and cryptochrome DASH (LmjF09.0360), which in some species bear 5,10-methenyltetrahydrofolate chromophores (Brudler, et al. 2003, Sancar 2004). Previously we obtained preliminary data suggesting the presence of a DNA photolyase repair activity in *L. major*, with UV-treated parasites showing a 2-fold enhancement in survival when allowed to recover while illuminated by visible light, compared to in darkness (data not shown). Thus we tested sensitivity to mutagens such as N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) or UV light towards *L. major*; however these were unaffected by PTR1 levels (Table 1). Consistent with this, studies described in following section did not implicate folates in oxidant susceptibility in *Leishmania*.

Taken together, the results in Table 1 strongly suggest that the role of PTR1 is limited to protection against reactive oxidants species (ROS) or the repair of oxidative damage.

PTR1-dependent modulation of H₂O₂ toxicity with exogenous biopterin

Several studies suggest exogenous pteridines can act oppositely in either mitigating or elevating oxidative stress depending upon circumstances (Oettl and Reibnegger 2002). The availability of the *ptr1*⁻ null mutant and *PTR1* overexpressor, coupled with the ability to control intracellular pteridine levels through supplementation and uptake via the *BT1* biopterin transporter (Cunningham and Beverley 2001, Ouellette, et al. 2002), allowed us to stringently assess the intracellular role of pteridines in *Leishmania*. In these experiments, parasites were tested in folate-deficient media (fdM199) containing various amounts of biopterin or H₂B; under all conditions parasites grew normally.

Since *ptr1*⁻ *L. major* requires H₂B for growth, biopterin-dependency was tested only with WT and *PTR1* overexpressors. WT parasites cultured without biopterin were highly susceptible to H₂O₂, with an EC₅₀ of ~2 μM (Fig. 2A). With increasing biopterin, the H₂O₂ EC₅₀ rose nearly 100-fold, plateauing at 150 μM H₂O₂ at concentrations above 840 nM biopterin (200 ng/ml; Fig. 2A, inset). In contrast, the *PTR1* overexpressor was much less sensitive to H₂O₂ at all biopterin concentrations tested, with an EC₅₀ of 15 μM H₂O₂ in the absence of biopterin supplementation, increasing to 250 μM above 211 nM biopterin (50 ng/ml, Fig. 2A). At 42 nM biopterin (10 ng/ml), a concentration which can support growth indefinitely, the difference in H₂O₂ EC₅₀s between WT and the *PTR1* overexpressor was 10-fold (Fig. 2A). Conversely, an EC₅₀ of 50 μM H₂O₂ was obtained with only 10 nM biopterin with the *PTR1* overexpressor, versus 211 nM for the WT.

Similar results were obtained in tests with H₂B supplementation (Fig. 2B). WT parasites showed a dose-dependent increase in H₂O₂ resistance, plateauing above 210 nM H₂B at an EC₅₀ of 150 µM H₂O₂. In contrast, *ptr1*⁻ parasites were much more sensitive to H₂O₂ at all concentrations tested, plateauing at 1.05 µM H₂B with an EC₅₀ of only 40 µM H₂O₂. Correspondingly, the PTR1 overexpressor was more resistant at all concentrations, plateauing at 210 nM H₂B with an EC₅₀ of 300 µM H₂O₂ (Fig. 2B). At 42 nM H₂B (10 ng/ml), WT showed an EC₅₀ of 40 µM H₂O₂, *ptr1*⁻ an EC₅₀ of 5 µM, and the PTR1 overexpressor an EC₅₀ of 150 µM. Conversely, an EC₅₀ of 50 µM was obtained at a concentration of 1 µM H₂B for the *ptr1*⁻ line, 42 nM for WT, and less than 4 nM for the PTR1 overexpressor (Fig. 2B).

Thus, biopterin supplementation invariably increased H₂O₂ resistance – an effect that was dramatically enhanced by PTR1 overexpression and abolished in its absence. These data suggest that PTR1 protects against oxidative stress by providing *Leishmania* with reduced biopterin and specifically H₄B intracellularly.

Alterations in DHFR-TS or folate levels do not affect H₂O₂ susceptibility

While PTR1 can reduce both biopterin and folate, DHFR is only able to reduce folates (Bello, et al. 1994, Nare, et al. 1997a). Unlike the PTR1 null or overexpressors, the H₂O₂ sensitivities a *dhfr-ts*⁻ null mutant or a DHFR-TS overexpressor were similar to that of WT (Fig. 3). Furthermore, *Leishmania* killing by H₂O₂ was unaffected by varying external folate levels (not shown). Thus, neither reduced folates nor DHFR-TS activity could be implicated in H₂O₂ resistance.

PTR1-dependent oxidant susceptibility does not arise through increased detoxification of H₂O₂

Pteridines can react directly with ROS, suggesting the possibility that reduced pteridines formed by PTR1 mediated susceptibility directly by detoxification, or ‘interception’ (Oettl and Reibnegger 2002). Thus we asked whether oxidant consumption was altered in a PTR1-dependent manner. The rate of extracellular H₂O₂ consumption by WT *Leishmania* was rapid (initial rates of 4 - 5 nmol/min/10⁸ cells), but no differences between WT, *ptr1*⁻ and the PTR1 overproducer were observed (Fig. 4A). Controls showed that H₂O₂ consumption was eliminated by boiling or sonicating the parasites prior to assay (data not shown), ruling out media effects and showing that H₂O₂ was only consumed by viable organisms. H₂O₂ was also stable in the absence of cells, and pre-incubation of *Leishmania* with high concentrations of biopterin (5-10 µg/ml) had no effect on the rate of H₂O₂ consumption (not shown). As a positive control, WT *L. major* pre-loaded with bovine catalase showed elevated rates of H₂O₂ consumption (Fig. 4A).

These results on the rate of H₂O₂ consumption were not unexpected, since intracellular H₄B levels are far below that of cellular thiols such as trypanothione (<10 µM v. 0.4 – 2 mM; Cunningham and Beverley 2001, Fairlamb and Cerami 1992, Moutiez, et al. 1994). Potentially, reduced pteridines could play a role in scavenging H₂O₂ if they were significantly more reactive than thiols. However, no data support this idea (Oettl and Reibnegger 2002, Werner-Felmayer, et al. 2002) and we did not find significant differences in the reactivity of H₂O₂ with various pteridines and thiols (Fig. 5).

PTR1-dependent oxidant susceptibility is not associated with decreased levels of intracellular oxidative stress

The H₂O₂ consumption assay does not directly measure the level of intracellular oxidative stress. Thus we employed the fluorescent dye 2',7'-dichlorofluorescein diacetate (DCFH-DA) to measure oxidants within the cell; following uptake this dye is metabolized and develops

elevated fluorescence when exposed to ROS (Robinson, et al. 1988). *L. major* were loaded with 5-10 μ M DCFH-DA, exposed to H_2O_2 and then oxidative stress assessed by this method. Under these conditions, H_2O_2 exposure increased DCF fluorescence in a dose-dependent manner (Fig. 4B). Remarkably, the *ptr1*⁻ mutant and *PTR1*-overexpressor behaved identically to WT (Fig. 4B). This was not changed in the presence of high levels of biopterin (5 μ g/ml) that enhanced PTR1-dependent survival after H_2O_2 exposure (Fig. 2; data not shown). Flow cytometry of DCFH-DA loaded and H_2O_2 treated *Leishmania* revealed a homogeneous fluorescence distribution for each cell population, with no difference between *ptr1*⁻ and *PTR1*-overexpressing lines (not shown). As a negative control, WT parasites were loaded with catalase and DCFH-DA; as expected these parasites showed greatly reduced fluorescence in response to H_2O_2 treatment (Fig. 4B).

These data showed that despite having a 20-fold difference in H_2O_2 susceptibility, *ptr1*⁻ and *PTR1*-overexpressing lines show similar levels of intracellular oxidative stress across a wide range of external H_2O_2 concentrations.

PTR1-mediated resistance confers increased H_2O_2 survival

Since H_4B depletion can slow parasite growth under some circumstances (Bello, et al. 1994), oxidation of this pteridine might suppress parasite growth without impairing parasite survival. Short-term cellular viability was therefore monitored using an MTT reduction assay. Exposure of WT *L. major* to 250 or 500 μ M H_2O_2 reduced the viability of cells in a dose-dependent manner, with 500 μ M peroxide killing $70 \pm 3\%$ of the WT parasites (Fig. 6). In contrast, *PTR1* overexpressors showed increased survival, with 500 μ M H_2O_2 killing only $41 \pm 4\%$ of the parasites. This indicates that although *PTR1* overexpression does not reduce the intracellular level of ROS, it nonetheless enhances short-term survival of oxidative stress.

DISCUSSION

PTR1* is an oxidant resistance gene *in vitro

Here we showed by several independent approaches that *Leishmania* pteridine reductase *PTR1* plays an important role in mediating resistance to oxidative stress. First, genetic selections for loci mediating resistance when over-expressed led to the recovery of episomal cosmid bearing *PTR1*, embedded within a region of the *Leishmania* chromosome designated the H region (Beverley, et al. 1984). The H region is notable because it has been found to be amplified independently in a number of *Leishmania* strains and species, in response to selection for resistance to an array of diverse agents including antifolates, sterol synthesis inhibitors, primaquine, and arsenicals and antimonial derivatives (Beverley 1991, Ouellette, et al. 2004). This initially suggested to some workers that the H region amplification represents one kind of nonspecific 'stress' response. However, subsequent studies have shown this model to be incorrect, as resistance to each agent arises from specific elements encoded within the H region, eg. *PTR1* and antifolates, metals and the P-glycoprotein *PGPA/MRP* (Callahan and Beverley 1991, Ouellette, et al. 1994), and terbinafine with *HTBF* (Marchini, et al. 2003). We extended this model here to show that resistance to H_2O_2 and a wide array of compounds that produce oxidative stress (including primaquine) is attributable specifically to *PTR1*. This was shown by *in vitro* generated transposon inactivation of *PTR1* within the cHP/H region cosmid (Fig. 1), as well as studies of isogenic *L. major* specifically lacking or overexpressing *PTR1* (Table 1).

One advantage of this selection strategy is that it allows the parasite to select the critical gene (s) without assumptions about the identity of these genes. Potentially a number of loci could mediate H_2O_2 resistance via overexpression, although here only the *PTR1* locus was recovered. Interestingly, overproduction of trypanothione reductase, the enzyme that maintains the T

[SH]₂ pool, failed to protect *Leishmania* against H₂O₂-mediated oxidative stress (Kelly, et al. 1993). This suggests that unlike the generation of H₄B, the rate of trypanothione reduction is not generally limiting in these parasites, even under oxidative stress, perhaps explaining why this key antioxidant enzyme was not recovered in our over-expression based screen. It is possible that other methods for placing and/or selecting *Leishmania* under oxidative stress would yield other loci, particularly since our screen was not exhaustive and did not yield evidence of genetic saturation.

PTR1 mediates oxidant resistance through provision of H₄B intracellularly

Our studies on parasite lines lacking or overexpressing *PTR1* showed a strong correlation between the level of this enzyme and resistance to a range of oxidative stresses. The diversity of the agents used renders unlikely the possibility that the resistance arises through 'off-target' effects unrelated to oxidative stress. Our data show that PTR1 mediates oxidant resistance through provision of H₄B, as the H₂O₂ susceptibility of *Leishmania* could be strongly modulated by varying external biopterin or H₂B. Importantly, this effect was strongly *PTR1*-dependent, establishing that it occurs intracellularly, unlike many studies of oxidative stress where pteridines were provided externally. In contrast, folates, although good PTR1 substrates, failed to modulate the susceptibility of *Leishmania* to H₂O₂, nor did the loss or overexpression of *DHFR-TS* (Nare, et al. 1997a). Previous studies of the phenotypes of *PTR1* and *DHFR-TS* knockouts and overexpressors (Bello, et al. 1994, Cruz, et al. 1991, Nare, et al. 1997a) indicated that unconjugated pteridines have uncharacterized functions in *Leishmania*, one of which we now assign as oxidant resistance. Interestingly, amplification of the H region in response to primaquine, a compound thought to act via the production of ROS (Augusto, et al. 1986), led to the suggestion that one of the H region proteins and possibly *PTR1* were implicated in oxidant resistance (Bello, et al. 1994, Ellenberger and Beverley 1989).

In contrast, damage mediated by agents solely generating RNS (acidified nitrite, nitrosoglutathione) was not modulated by *PTR1*, suggesting that reduced unconjugated pteridines are not involved in defense against nitric oxide and its metabolites in *Leishmania*. This contrasts to the action of the thiol-based antioxidants in *Leishmania*, such as ovoidthiol and the peroxiredoxins, which are active against both ROS and RNS (Iyer, et al. 2008, Vogt and Steenkamp 2003). This is another argument against a detoxification model for PTR1-mediated ROS resistance, as discussed below. Interestingly, PTR1 did provide some protection against killing by SIN-1, a generator of both of NO and O₂⁻ (Feelisch, et al. 1989). The differential toxicity of SIN-1 between *ptr1*⁻ and the PTR1-overproducer most parsimoniously reflects the effects of O₂⁻, which will be converted to H₂O₂ by the *Leishmania* superoxide dismutase (Pleues, et al. 2003). Alternatively, PTR1 could mediate resistance to peroxynitrite formed from the reaction of SIN-1 generated NO and O₂⁻ (Stamler 1994), as peroxynitrite is toxic to *Leishmania in vitro* (Denicola, et al. 1993, Gatti, et al. 1995), and H₄B has been shown to react with peroxynitrite (Milstien and Katusic 1999). Future studies will be required to establish whether PTR1 and H₄B affect the peroxynitrite sensitivity of *Leishmania*.

PTR1-dependent susceptibility to oxidants does not correlate with increased oxidant detoxification

The data presented establish that PTR1 mediates resistance to ROS through H₄B. There are several ways that H₄B might protect against oxidative stress. First, while H₄B is known to induce oxidative stress through Fenton chemistry (Kirsch, et al. 2003), the complexities of pteridine/oxidant interactions (Oettl and Reibnegger 2002) conspire to make it difficult to rule out the possibility that under some circumstances scavenging might occur. However, in tests of lines lacking or overexpressing PTR1, and showing nearly 20-fold differential H₂O₂ sensitivity, H₂O₂ metabolism was similar, as was the degree of intracellular oxidative stress elicited by H₂O₂ treatment (Fig. 4); neither result would be expected if H₄B did exert direct

protective or oxidative effects. A lack of cross-resistance to pure RNS stress is a second argument against a direct detoxification model, as noted above (Table 1). Although direct oxidant-pteridine reactions undoubtedly occur within the cell, they apparently contribute little to the mechanism of oxidative resistance described here.

Thus we conclude that PTR1/biopterin dependent oxidative resistance does not arise through a detoxification mechanism in *Leishmania*, which instead is the responsibility of the well known trypanothione-dependent antioxidant systems of *Leishmania* and other trypanosomatids (Krauth-Siegel, et al. 2007, Krauth-Siegel, et al. 2003). This conclusion is also consistent with the >100-fold higher concentrations of cellular thiols relative to unconjugated pteridines, with T[SH]₂ ranging from at 0.4-2 mM (Fairlamb and Cerami 1992, Moutiez, et al. 1994) while H₄B levels are typically less than 10 μM (Cunningham and Beverley 2001). These calculations suggest that at most the presence or absence of H₄B would alter the cellular concentration of potential oxidant scavenging species by < 1%. While it is theoretically possible that the concentration differential could be balanced by hyper-reactivity of pteridines with oxidants, current data suggest this is unlikely (Fig. 5; refs. Oettl and Reibnegger 2002, Werner-Felmayer, et al. 2002).

While our manuscript was under review, Moreira *et al* presented data similarly implicating PTR1 in H₂O₂ susceptibility (Moreira, et al. 2009), albeit with some important differences. In contrast to our findings (Table 1), their *ptr1*⁻ mutants showed ~2-fold increased susceptibility to SNAP, although the PTR1-overexpressors resembled WT. SNAP has the potential to generate peroxynitrite (Huang, et al. 2005), which as noted in our studies of SIN-1 might be targeted by PTR1-dependent activities (Table 1). Notably, 'pure' RNS generators such as acidified nitrite or S-nitrosoglutathione revealed no resistance (Table 1). Secondly, Moreira *et al* inferred a role for increased scavenging in comparisons of WT v. *ptr1*⁻ mutants. The effect was modest (<2 fold), and little effect was seen in the PTR1 overexpressors despite their increased H₂O₂ resistance. The difference in these studies may be attributable to the experimental methods used; in our study DCFH-DA loaded parasites were challenged after 15 min, with a range of H₂O₂ concentrations and followed over time. In contrast, Moreira *et al* maintained parasites 45 min in buffered saline, which were then exposed to oxidants for a further 45 min prior to addition of DHCH-DA, and a single measurement taken. This prolonged incubation, under stressful conditions of nutrient deprivation, may have perturbed cellular metabolism. We believe our data, in combination with studies showing no increase in H₂O₂ consumption and the overwhelming 100-fold excess of thiols such a trypanothione, argue strongly against a significant contribution by oxidant scavenging to PTR1-dependent oxidant resistance.

What is the mechanism of PTR1-mediated oxidant resistance?

The known role of PTR1 is to maintain H₄B pools, as this metabolite is required for growth (Bello, et al. 1994, Nare, et al. 1997a). Since the reason why H₄B is required in *Leishmania* metabolism is not yet known, specific tests for the role of H₄B-dependent enzymes in oxidant resistance are not presently feasible. A major challenge in such attempts to define the functions of reduced pteridines is the relative scarcity and instability of these compounds. One model consistent with the data presented here implicates reduced unconjugated pteridines in the recovery of *Leishmania* from damage by ROS, either by directly repairing oxidative damage to cellular components, or indirectly by maintaining cellular pathways affecting oxidant susceptibility. Organisms possess many pathways for the repair of oxidative damage to proteins, lipids and nucleic acids (Davies 1995), and perhaps H₄B participates in the reversal of oxidant damage to any of these macromolecules (Oettl and Reibnegger 2002, Werner-Felmayer, et al. 2002), or in other cellular protective responses.

H₄B mediated oxidant resistance, gene amplification and *Leishmania* virulence

Since the ability of pathogens to withstand oxidative stress is often important to infectivity (Fang 2004), it seems likely that PTR1-dependent oxidant susceptibility could be important to *Leishmania* survival and virulence, especially within mammalian macrophages. For example, decreased expression of trypanothione reductase reduces survival within macrophages (Dumas, et al. 1997), and studies of the abundant *Leishmania* surface glycoconjugate lipophosphoglycan have shown that even 2-fold alterations in oxidant sensitivity have strong effects on parasites' ability to survive within macrophages (Spath, et al. 2003).

One argument against a significant role in virulence is the fact that the H₂O₂ sensitive *ptr1*⁻ *L. major* CC-1 line studied here is able to infect susceptible mice (Cunningham, et al. 2001). However, the relationship between oxidative susceptibility measured *in vitro*, and its relevancy to virulence of pathogens *in vivo*, is often complex, depending on the pathogen and specific gene tested. For example, catalase-deficient mutants of *Haemophilus influenzae* show little effect on virulence in animal models, despite increased oxidant susceptibility *in vitro* (Bishai, et al. 1994, Vergauwen, et al. 2006). A further complication is the fact that the CC-1 line shows somewhat attenuated in mouse infectivity studies relative to fully-virulent lines, probably the result of prolonged cultivation *in vitro* (Titus, et al. 1995). Lastly, the CC-1 strain, like many laboratory-adapted strains, shows significant alterations in pteridine metabolism (Cruz and Beverley 1990, Cruz, et al. 1993, Roy, et al. 2001). Thus, an assessment of the role(s) of reduced pteridines and oxidative stress in the *Leishmania* infectious cycle will need to be carried out in fully-virulent strains, in both sand flies, mice and macrophages.

Leishmania as a platform for studying the role of pteridines and susceptibility to oxidative stress

More generally, *L. major* provides an excellent model for studies aimed at dissecting the potential role of pteridines in resistance to oxidative stress *in vivo*. As shown here and elsewhere, the potent salvage pathways of *Leishmania* facilitate the manipulation of intracellular pteridine levels through nutritional supplementation, as do the availability of lines lacking or overexpressing critical pteridine metabolic enzymes such as PTR1, DHFR-TS and other folate dependent pathways (Cunningham and Beverley 2001, Nare, et al. 1997a, Roy, et al. 2001). In combination, these features provide a unique ability to manipulate the intracellular levels of reduced pteridines. This is especially relevant to studies of pteridine-mediated oxidant resistance in mammalian cells, which has proven to be exceedingly complex (reviewed in ref. Oettl and Reibnegger 2002). *Leishmania* thus provides a unique opportunity to probe the role of pteridines in oxidant resistance in a comparatively simple and readily-manipulated experimental system.

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References

- Ariyanayagam MR, Fairlamb AH. Ovothiol and trypanothione as antioxidants in trypanosomatids. *Mol Biochem Parasitol* 2001;115:189–198. [PubMed: 11420105]
- Ashtosh, Sundar S, Goyal N. Molecular mechanisms of antimony resistance in *Leishmania*. *J Med Microbiol* 2007;56:143–153. [PubMed: 17244793]

- Augusto O, Alves MJM, Colli W, Filardi LS, Brener S. Primaquine can mediate hydroxyl radical generation by *Trypanosoma cruzi* extracts. *Biochemical and Biophysical Research Communication* 1986;135:1029–1034.
- Bello AR, Nare B, Freedman D, Hardy L, Beverley SM. PTR1: a reductase mediating salvage of oxidized pteridines and methotrexate resistance in the protozoan parasite *Leishmania major*. *Proceedings of the National Academy of Sciences* 1994;91:11442–11446.
- Beverley SM. Gene Amplification in *Leishmania*. *Annu Rev Microbiol* 1991;45:417–444. [PubMed: 1741620]
- Beverley, SM. Genetic and genomic approaches to the analysis of *Leishmania* virulence. In: Marr, JM.; Nilsen, T.; Komuniecki, R., editors. *Molecular & Medical Parasitology*. Academic Press; New York: 2003.
- Beverley SM, Coderre JA, Santi DV, Schimke RT. Unstable DNA amplifications in methotrexate resistant *Leishmania* consist of extra-chromosomal circles which relocate during stabilization. *Cell* 1984;38:431–439. [PubMed: 6467372]
- Bishai WR, Howard NS, Winkelstein JA, Smith HO. Characterization and virulence analysis of catalase mutants of *Haemophilus influenzae*. *Infection and immunity* 1994;62:4855–4860. [PubMed: 7927766]
- Brudler R, Hitomi K, Daiyasu H, Toh H, Kucho K, Ishiura M, Kanehisa M, Roberts VA, Todo T, Tainer JA, Getzoff ED. Identification of a new cryptochrome class. Structure, function, and evolution. *Mol Cell* 2003;11:59–67. [PubMed: 12535521]
- Callahan HL, Beverley SM. Heavy metal resistance: a new role for P-glycoproteins in *Leishmania*. *Journal of Biological Chemistry* 1991;266:18427–18430. [PubMed: 1680861]
- Clos J, Choudhury K. Functional cloning as a means to identify *Leishmania* genes involved in drug resistance. *Mini reviews in medicinal chemistry* 2006;6:123–129. [PubMed: 16472181]
- Cotrim PC, Garrity LK, Beverley SM. Isolation of genes mediating resistance to inhibitors of nucleoside and ergosterol metabolism in *Leishmania* by overexpression/selection. *J Biol Chem* 1999;274:37723–37730. [PubMed: 10608831]
- Cruz A, Beverley SM. Gene replacement in parasitic protozoa. *Nature* 1990;348:171–173. [PubMed: 2234081]
- Cruz A, Coburn C, Beverley SM. Double targeted gene replacement for creating null mutants. *Proceedings of the National Academy of Sciences* 1991;88:7170–7174.
- Cruz AK, Titus R, Beverley SM. Plasticity in chromosome number and testing of essential genes in *Leishmania* by targeting. *Proc Natl Acad Sci U S A* 1993;90:1599–1603.
- Cunningham ML, Beverley SM. Pteridine salvage throughout the *Leishmania* infectious cycle: implications for antifolate chemotherapy. *Mol Biochem Parasitol* 2001;113:199–213. [PubMed: 11295174]
- Cunningham ML, Titus RG, Turco SJ, Beverley SM. Regulation of differentiation to the infective stage of the protozoan parasite *Leishmania major* by tetrahydrobiopterin. *Science* 2001;292:285–287. [PubMed: 11303103]
- Davies KJ. Oxidative stress: the paradox of aerobic life. *Biochem Soc Symp* 1995;61:1–31. [PubMed: 8660387]
- Denicola A, Rubbo H, Rodriguez D, Radi R. Peroxynitrite-mediated cytotoxicity to *Trypanosoma cruzi*. *Archives of Biochemistry and Biophysics* 1993;304:279–286. [PubMed: 8323293]
- Denkers EY, Butcher BA. Sabotage and exploitation in macrophages parasitized by intracellular protozoans. *Trends Parasitol* 2005;21:35–41. [PubMed: 15639739]
- Desjeux P. Leishmaniasis: current situation and new perspectives. *Comp Immunol Microbiol Infect Dis* 2004;27:305–318. [PubMed: 15225981]
- Devine SE, Boeke JD. Efficient intergration of artificial transposons into plasmid targets in vitro: a useful tool for DNA mapping, sequencing and genetic analysis. *Nucleic acid research* 1994;22:3765–3772.
- Dumas C, Ouellette M, Tovar J, Cunningham ML, Fairlamb AH, Tamar S, Olivier M, Papadopoulos B. Disruption of the trypanothione reductase gene of *Leishmania* decreases its ability to survive oxidative stress in macrophages. *Embo J* 1997;16:2590–2598. [PubMed: 9184206]
- Ellenberger TE, Beverley SM. Multiple drug resistance and conservative amplification of the H region in *Leishmania major*. *Journal of Biological Chemistry* 1989;264:15094–150103. [PubMed: 2768255]

- Fairlamb AH, Cerami A. Metabolism and functions of trypanothione in the kinetoplastida. *Annual Review of Microbiology* 1992;46:695–729.
- Fang FC. Antimicrobial reactive oxygen and nitrogen species: concepts and controversies. *Nature reviews* 2004;2:820–832.
- Feelisch M, Ostrowski J, Noack E. On the mechanism of NO release from sydnonimines. *Journal of Cardiovascular Pharmacology* 1989;14:S13–22. [PubMed: 2484692]
- Garraway LA, Tosi LRO, Wang Y, Moore JB, Dobson DE, Beverley SM. Insertional mutagenesis using a modified in vitro Ty1 transposition system. *Nature Biotechnology*. 1997
- Gatti RM, Augusto O, Kwee JK, Giorgio S. Leishmanicidal activity of peroxynitrite. *Redox Report* 1995;1:261–265.
- Homan-Muller JWT, Weening RT, Roos D. Production of hydrogen peroxide by phagocytizing human granulocytes. *Journal of Laboratory and Clinical Medicine* 1975;85:198–207. [PubMed: 1113012]
- Huang HM, Chen HL, Xu H, Gibson GE. Modification of endoplasmic reticulum Ca²⁺ stores by select oxidants produces changes reminiscent of those in cells from patients with Alzheimer disease. *Free radical biology & medicine* 2005;39:979–989. [PubMed: 16198225]
- Iyer JP, Kaprakkaden A, Choudhary ML, Shaha C. Crucial role of cytosolic tryparedoxin peroxidase in *Leishmania donovani* survival, drug response and virulence. *Mol Microbiol* 2008;68:372–391. [PubMed: 18312262]
- Jaeger T, Flohe L. The thiol-based redox networks of pathogens: unexploited targets in the search for new drugs. *Biofactors* 2006;27:109–120. [PubMed: 17012768]
- Kapler GM, Coburn CM, Beverley SM. Stable transfection of the human parasite *Leishmania major* delineates a 30-kilobase region sufficient for extrachromosomal replication and expression. *Molecular & Cellular Biology* 1990;10:1084–1094. [PubMed: 2304458]
- Kaufman S. The structure of phenylalanine hydroxylation cofactor. *Proceedings of the National Academy Sciences* 1963;50:1085–1093.
- Kelly JM, Taylor MC, Smith K, Hunter KJ, Fairlamb AH. Phenotype of recombinant *Leishmania donovani* and *Trypanosoma cruzi* which over-express trypanothione reductase. Sensitivity towards agents that are thought to induce oxidative stress. *European Journal of Biochemistry* 1993;218:29–37. [PubMed: 8243474]
- Keston AS, Brandt R. The fluorimetric analysis of ultramicroquantities of hydrogen peroxide. *Analytical Biochemistry* 1965;11:1–5. [PubMed: 14328641]
- Kirsch M, Korth HG, Stenert V, Sustmann R, de Groot H. The autoxidation of tetrahydrobiopterin revisited. Proof of superoxide formation from reaction of tetrahydrobiopterin with molecular oxygen. *J Biol Chem* 2003;278:24481–24490. [PubMed: 12714605]
- Kosar-Hashemi B, Armarego WLF. A convenient spectrophotometric method for measuring the kinetic parameters of glyceryl-ether monooxygenase (EC1.14.16.5). *Biological Chemical Hoppe-Seyler* 1993;374:9–25.
- Krauth-Siegel LR, Comini MA, Schlecker T. The trypanothione system. *Subcell Biochem* 2007;44:231–251. [PubMed: 18084897]
- Krauth-Siegel RL, Ludemann H. Reduction of dehydroascorbate by trypanothione. *Mol Biochem Parasitol* 1996;80:203–208. [PubMed: 8892297]
- Krauth-Siegel RL, Meiering SK, Schmidt H. The parasite-specific trypanothione metabolism of *Trypanosoma* and *Leishmania*. *Biol Chem* 2003;384:539–549. [PubMed: 12751784]
- Liew FY, Millott S, Parkinson C, Palmer RMJ, Moncada S. Macrophage killing of *Leishmania* parasite in vivo is mediated by nitric oxide from L-arginine. *Journal of Immunology* 1990;144:4794–4797.
- Ma D, Beverley SM, Turco SJ. *Leishmania donovani* possess a NADPH-dependent alkylglyceryl cleavage enzyme. *Biochemical Biophysical Research Communication* 1996;227:885–889.
- Marchini JF, Cruz AK, Beverley SM, Tosi LR. The H region HTBF gene mediates terbinafine resistance in *Leishmania major*. *Mol Biochem Parasitol* 2003;131:77–81. [PubMed: 12967714]
- Milstien S, Katusic Z. Oxidation of tetrahydrobiopterin by peroxynitrite: implications for vascular endothelial function. *Biochem Biophys Res Commun* 1999;263:681–684. [PubMed: 10512739]

- Moreira W, Leblanc E, Ouellette M. The role of reduced pterins in resistance to reactive oxygen and nitrogen intermediates in the protozoan parasite *Leishmania*. *Free radical biology & medicine* 2009;46:367–375. [PubMed: 19022374]
- Moutiez M, Meziane-Cherif D, Aumercier M, Sergheraert C, Tartar A. Compared reactivities of trypanothione and glutathione in conjugation reactions. *Chemical Pharmacology Bulletin* 1994;42:2641–2644.
- Nare B, Hardy L, Beverley SM. The roles of pteridine reductase 1 (PTR1) and dihydrofolate reductase-thymidylate synthase (DHFR-TS) in pteridine metabolism in the protozoan parasite *Leishmania major*. *Journal of Biological Chemistry* 1997a;272:13883–13891. [PubMed: 9153248]
- Nare B, Luba J, Hardy LW, Beverley S. New approaches to *Leishmania* chemotherapy: pteridine reductase 1 (PTR1) as a target and modulator of antifolate sensitivity. *Parasitology* 1997b;114:S101–110. [PubMed: 9309772]
- Oettl K, Reibnegger G. Pteridine derivatives as modulators of oxidative stress. *Curr Drug Metab* 2002;3:203–209. [PubMed: 12003351]
- Ouellette M, Drummelsmith J, El-Fadili A, Kundig C, Richard D, Roy G. Pterin transport and metabolism in *Leishmania* and related trypanosomatid parasites. *Int J Parasitol* 2002;32:385–398. [PubMed: 11849635]
- Ouellette M, Drummelsmith J, Papadopolou B. Leishmaniasis: drugs in the clinic, resistance and new developments. *Drug Resist Updat* 2004;7:257–266. [PubMed: 15533763]
- Ouellette M, Legare D, Papadopolou B. Microbial multidrug-resistance ABC transporters. *Trends in Microbiology* 1994;2:407–411. [PubMed: 7850210]
- Penketh PGAK RA. Hydrogen peroxide metabolism in *Trypanosoma brucei*. *Molecular and Biochemical Parasitology* 1986;20:111–121. [PubMed: 3748070]
- Pick E, Keisari Y. A simple calorimetric assay for the measurement of hydrogen peroxide produced by cells in culture. *Journal of Immunological Methods* 1980;38:161–170. [PubMed: 6778929]
- Plewes KA, Barr SD, Gedamu L. Iron superoxide dismutases targeted to the glycosomes of *Leishmania chagasi* are important for survival. *Infection and immunity* 2003;71:5910–5920. [PubMed: 14500512]
- Robinson JP, Bruner LH, Bassoe CF, Hudson JL, Ward PA, Phan SH. Measurement of intracellular fluorescence of human monocytes relative to oxidative metabolism. *Journal of Leucocyte Biology* 1988;43:304–310.
- Roy G, Kundig C, Olivier M, Papadopolou B, Ouellette M. Adaptation of *Leishmania* cells to in vitro culture results in a more efficient reduction and transport of bipterin. *Exp Parasitol* 2001;97:161–168. [PubMed: 11312578]
- Ryan KA, Dasgupta S, Beverley SM. Shuttle cosmid vectors for the trypanosomatid parasite *Leishmania*. *Gene* 1993;131:145–150. [PubMed: 8370535]
- Sancar A. Photolyase and cryptochrome blue-light photoreceptors. *Adv Protein Chem* 2004;69:73–100. [PubMed: 15588840]
- Spath GF, Garraway LA, Turco SJ, Beverley SM. The role(s) of lipophosphoglycan (LPG) in the establishment of *Leishmania* major infections in mammalian hosts. *Proc Natl Acad Sci U S A* 2003;100:9536–9541. [PubMed: 12869694]
- Stamler JS. Redox signalling: Nitrosylation and related target interactions of nitric oxide. *Cell* 1994;78:931–936. [PubMed: 7923362]
- Tayeh MA, Marletta MA. Macrophage oxidation of L-arginine to nitric oxide, nitrite and nitrate. *Journal of Biological Chemistry* 1989;264:19654–19658. [PubMed: 2584186]
- Titus RG, Gueiros-Filho FJ, de Freitas LA, Beverley SM. Development of a safe live *Leishmania* vaccine line by gene replacement. *Proceedings of the National Academy of Sciences of the United States of America* 1995;92:10267–10271. [PubMed: 7479765]
- Vergauwen B, Herbert M, Van Beeumen JJ. Hydrogen peroxide scavenging is not a virulence determinant in the pathogenesis of *Haemophilus influenzae* type b strain Eagan. *BMC microbiology* 2006;6:3. [PubMed: 16430767]
- Vogt RN, Steenkamp DJ. The metabolism of S-nitrosothiols in the trypanosomatids: the role of ovothiol A and trypanothione. *Biochem J* 2003;371:49–59. [PubMed: 12487629]

- Wanasen N, Soong L. L-arginine metabolism and its impact on host immunity against *Leishmania* infection. *Immunol Res* 2008;41:15–25. [PubMed: 18040886]
- Wang J, Leblanc E, Chang CF, Papadopoulou B, Bray T, Whiteley JM, Lin SX, Ouellette M. Pterin and folate reduction by the *Leishmania tarentolae* H locus short- chain dehydrogenase/reductase PTR1. *Arch Biochem Biophys* 1997;342:197–202. [PubMed: 9186479]
- Werner-Felmayer G, Golderer G, Werner ER. Tetrahydrobiopterin biosynthesis, utilization and pharmacological effects. *Curr Drug Metab* 2002;3:159–173. [PubMed: 12003348]
- Wyllie S, Cunningham ML, Fairlamb AH. Dual action of antimonial drugs on thiol redox metabolism in the human pathogen *Leishmania donovani*. *J Biol Chem* 2004;279:39925–39932. [PubMed: 15252045]

Abbreviations

PTR1	pteridine reductase 1
DHFR-TS	dihydrofolate reductase-thymidylate synthase
ROS	reactive oxygen species
RNS	reactive nitrogen species
DCFH-DA	2'7'-dichlorofluorescein diacetate
DCF	dichlorofluorescein
GSH	glutathione
T[SH]₂	trypanothione
SIN-1	3-morpholino-sydnnonimine
H₂B	dihydrobiopterin
H₄B	tetrahydrobiopterin
WT	wild-type

Figure 1. Multi-copy selection scheme and TyK transposition into cHP

A. A cosmid library-transfected parasite population was incubated in the presence of H_2O_2 to amplify a population containing cosmid-inserts that confer H_2O_2 resistance. Cosmid DNA was recovered from this population and, and genetic analysis identified the resistance gene.

B. Insertional mutagenesis was performed using the TyK artificial transposon and cHP insertion mutants were characterized by *Hind*III digestion or amplification of the *PTR1* coding region by polymerase chain reaction. Selected single insertions were transfected into *Leishmania* and H_2O_2 resistance was determined as described in the methods.

Figure 2. Growth inhibition by H₂O₂ and modulation with biopterin

Leishmania promastigotes ($2 \times 10^5 \text{ ml}^{-1}$) were incubated in fdM199 containing H₂O₂ (0 - 450 μM) in the presence of increasing concentrations of biopterin (Panel A) or H₂ B (Panel B). The inset in each panel shows the effects at higher concentrations. Growth inhibition was determined as described in the methods. (○) WT; (●) *ptr1*⁻/+PTR1; and (◇) *ptr1*⁻. This experiment was repeated three times, with similar results to the one shown.

Figure 3. Comparison of PTR1 and DHFR-TS in protection against oxidative damage
Leishmania (2×10^5 ml⁻¹) were incubated in fdM199 containing H₂O₂ (0 - 450 μM) in the presence of 10 ng/ml H₂B and 10 μg/ml thymidine. Growth inhibition was determined as described in the methods. (●) WT; (○) *ptr1*⁻; (▼) *ptr1*⁻/*+PTR1*; (Δ) *dhfr-ts*⁻; (■) *dhfr-ts*⁻/*+DHFR-TS*. For comparison with data included in Table 1, the EC₅₀s calculated for WT, *ptr1*⁻ and *ptr1*⁻/*+PTR1* in this experiment were 62, 7 and 140 μM, respectively. This experiment was repeated three times, with similar results to the one shown.

Figure 4. Metabolism of H₂O₂ and measurement of intracellular oxidative stress

A. Cells (1×10^7 /ml) were incubated in buffer containing 20 μ M H₂O₂ at zero time. Aliquots (1 ml) were withdrawn at indicated time intervals and extracellular H₂O₂ assayed as described in the methods. (●) No cells, (▼) WT; (■) *ptr1*⁻; (◇) *ptr1*⁻/*PTR1*; (▲) WT pre-loaded with catalase.

B. Cells were rinsed, resuspended in Hanks balanced salt solution (1×10^7 ml⁻¹) and loaded with 10 μ M DCF-DA for 15 min. Intracellular oxidant stress was determined by monitoring fluorescence due to oxidation of DCF-DA to DCF, in the presence of indicated amounts of H₂O₂ as described in the methods. (●) WT; (○) *ptr1*⁻; (▼) *ptr1*⁻/*PTR1*; (▲) WT pre-loaded with catalase. This experiment was repeated three times, with similar results to the one shown.

Figure 5. Comparison of the reactivity of H₂O₂ with pteridines and thiols

H₂O₂ (30 μM) was added to 1 ml buffer containing increasing concentration of freshly prepared pteridine or glutathione and allowed to react for 5 min at 30°C. Remaining H₂O₂ was quantified as described in the methods. (●) Biopterin; (○) H₂B; (Δ) H₄B; (▼) GSH. This experiment was repeated twice, with similar results to the one shown.

Figure 6. Effects of H₂O₂ on short-term cell viability in WT versus PTR1 overexpressors
Triplicate samples of *L. major* strain FV1 WT or *ptr1*⁻/SSU::*PTR1* cells were incubated at 8×10^6 cells ml⁻¹ in FDSL-M199 medium alone, or containing 250 or 500 μ M H₂O₂, for 2 h. Samples were then removed and the number of viable cells quantified using the MTT assay, as described in the methods. Data are expressed as a percentage of the amount of MTT reduced by the respective negative control for each cell line.

Table 1
Relationship between the PTR1 status of *Leishmania* and their resistance to stress conditions.

Stress treatment	Mode	WT EC ₅₀	<i>ptr1</i> ⁻ (KO) EC ₅₀	<i>ptr1</i> /WT	<i>ptr1</i> ⁺ / <i>PTR1</i> (OE) EC ₅₀	OE/WT	OE/KO
H ₂ O ₂ (μM)	ROS	32.8 ± 5.2	8.7 ± 1.9	0.27 **	157 ± 2	4.8 **	18 **
Glucose oxidase (mU)	ROS	0.4 ± 0.1	0.11 ± 0.02	0.28 **	0.5 ± 0.1	1.3 ns	5.0 **
<i>t</i> -butyl hydroperoxide (μM)	ROS	0.13 ± 0.02	0.13 ± 0.02	1.0 ns	0.4 ± 0.04	3.1 **	3.1 **
Primaquine (μM)	ROS	22 ± 3	5.9 ± 0.5	0.27 **	32 ± 2	1.5 **	5.4 **
Paraquat (mM)	ROS	1.6 ± 0.1	1.3 ± 0.2	0.81 ns	4.1 ± 0.9	2.6 **	3.2 **
3-morpholininosydininine (μM)	RNS+ROS	110 ± 20	32 ± 5	0.29 **	170 ± 70	1.5 ns	5.3 *
Na-nitrite (pH 7.2, mM)	RNS	2.4 ± 0.2	2.1 ± 0.5	0.88 ns	3.1 ± 0.4	1.3 ns	1.5 ns
Na-nitrite (pH 6.8, mM)	RNS	1.5 ± 0.4	1.2 ± 0.4	0.80 ns	1.5 ± 0.2	1.0 ns	1.3 ns
<i>S</i> -nitrosoacetylpenicillamine (μM)	RNS	49 ± 2	58 ± 3	1.18 *	37 ± 2	0.8 **	0.6 **
<i>S</i> -nitrosoglutathione (μM)	RNS	1.3 ± 0.2	1.2 ± 0.1	0.92 ns	1.4 ± 0.1	1.1 ns	1.2 ns
Allopurinol (μM)	Other	1.1 ± 0.5	1.9 ± 1.0	1.73 ns	1.4 ± 0.1	1.3 ns	0.7 ns
Terbinafine (μM)	Other	2.7 ± 0.3	3 ± 1	1.11 ns	3 ± 1	1.1 ns	1 ns
Pentamidine (μM)	Other	2.3 ± 0.7	2.6 ± 0.5	1.13 ns	2.6 ± 0.5	1.1 ns	1 ns
Nitroguanidine (μM)	Other	10 ± 3	11 ± 4	1.10 ns	11 ± 4	1.1 ns	1 ns
UV light (mJ cm ⁻¹)	Other	7 ± 2	6 ± 3	0.86 ns	6 ± 3	0.9 ns	1 ns

Results are EC₅₀s of 3-5 determinations in independent experiments, shown ± standard deviations. EC₅₀s are defined as the condition that decreases *Leishmania* major growth by 50%. KO = null mutant (*ptr1*⁻); OE = overexpressor (*ptr1*⁺/*PTR1*). Statistical tests were calculated first as between *ptr1*⁻ vs. WT, then the overexpressor (OE; *ptr1*⁺/*PTR1* vs. WT), and then of the ratio of the EC₅₀'s of the OE/KO.

** p <0.01;

* p <0.05 Student's unpaired two tailed t-test; calculated by algorithm implemented in GraphPad Software.