A comparative study of methylglyoxal metabolism in trypanosomatids

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The protozoan parasites Trypanosoma cruzi, Trypanosoma brucei and Leishmania spp. are the causative agents of the human infections Chagas’ disease, sleeping sickness and leishmaniasis, respectively. These diseases are responsible for more than 120 000 fatalities annually and the loss of over 4 600 000 disease-adjusted life-years [1]. Some of the poorest areas of the world are afflicted by these vector-borne parasites, and the accompanying economic burden is a major obstacle to improving human health [2]. Current treatments for protozoan diseases suffer from a range of problems, including severe toxic side effects [3] and acquired drug resistance [4,5]. To compound these difficulties, many of the current chemotherapeutic treatments require lengthy periods of hospitalization and are prohibitively expensive [1]. Therefore, novel drug targets and more effective drug treatments are required to combat these problems.

Metabolic pathways that are absent from, or significantly different to, host pathways are logical starting points for drug discovery [2,6]. Trypanosomatids possess NADPH-dependent methylglyoxal reductase and NAD⁺-dependent l-lactaldehyde dehydrogenase activities sufficient to account for all of the methylglyoxal metabolized by these cells. We propose that the predominant mechanism for methylglyoxal detoxification in the African trypanosome is via the methylglyoxal reductase pathway to l-lactate.

The glyoxalase system, comprising the metalloenzymes glyoxalase I (GLO1) and glyoxalase II (GLO2), is an almost universal metabolic pathway involved in the detoxification of the glycolytic byproduct methylglyoxal to l-lactate. In contrast to the situation with the trypanosomatid parasites Leishmania major and Trypanosoma cruzi, this trypanothione-dependent pathway is less well understood in the African trypanosome, Trypanosoma brucei. Although this organism possesses a functional GLO2, no apparent GLO1 gene could be identified in the T. brucei genome. The absence of GLO1 in T. brucei was confirmed by the lack of GLO1 activity in whole cell extracts, failure to detect a GLO1-like protein on immunoblots of cell lysates, and lack of l-lactate formation from methylglyoxal as compared to L. major and T. cruzi. T. brucei procycls were found to be 2.4-fold and 5.7-fold more sensitive to methylglyoxal toxicity than T. cruzi and L. major, respectively. T. brucei also proved to be the least adept of the ‘Tritryp’ parasites in metabolizing methylglyoxal, producing l-lactate rather than d-lactate. Restoration of a functional glyoxalase system by expression of T. cruzi GLO1 in T. brucei resulted in increased resistance to methylglyoxal and increased conversion of methylglyoxal to d-lactate, demonstrating that GLO2 is functional in vivo. Procyclic forms of T. brucei possess NADPH-dependent methylglyoxal reductase and NAD⁺-dependent l-lactaldehyde dehydrogenase activities sufficient to account for all of the methylglyoxal metabolized by these cells. We propose that the predominant mechanism for methylglyoxal detoxification in the African trypanosome is via the methylglyoxal reductase pathway to l-lactate.

Abbreviations
GLO1, glyoxalase I; GLO2, glyoxalase II; TcGLO1, Trypanosoma cruzi glyoxalase I.
mammalian hosts), which utilize glutathione (γ-L-glutamyl-L-cysteinylglycine) [7]. This dithiol is primarily responsible for the maintenance of thiol-redox homeostasis within trypanosomatids, and is crucially involved in the protection of parasites from oxidative stress [8], heavy metals [9] and xenobiotics [10]. Several enzymes involved in trypanothione biosynthesis and its downstream metabolism have been genetically and chemically validated as essential for parasite survival [11]. Consequently, trypanothione-dependent enzymes have become the focus of much anti-trypanosomatid drug discovery.

The glyoxalase system, comprising the metalloenzymes glyoxalase I (GLO1, EC 4.4.1.5) and glyoxalase II (GLO2, EC 3.1.2.6), together with glutathione as cofactor, is a widely distributed pathway involved in metabolism of the toxic and mutagenic glycolytic byproduct methylglyoxal [12,13]. A unique trypanothione-dependent glyoxalase system has been identified in Leishmania spp. and T. cruzi [14–16]. In the first step, GLO1 isomerizes the spontaneous hemithioacetal adduct formed between trypanothione and methylglyoxal to S-β-lactoyltrypanothione [14]. In the second step, GLO2 catalyses hydrolysis of this ester, releasing β-lactate and regenerating trypanothione. The trypanothione-dependent glyoxalase system in these parasites differs significantly from that employed by their mammalian hosts, which depends entirely on glutathione as a thiol cofactor. These differences in substrate specificity may provide an opportunity for the specific chemotherapeutic targeting of these enzymes in the trypanosomatids. As inhibitors of the glyoxalase system have already been shown to possess both anticancer and antimalarial [18] activities, it is possible that inhibition of the trypanothione-dependent glyoxalase pathway may prove toxic to trypanosomatids.

Although glyoxalase metabolism has been well defined in both Leishmania major and T. cruzi, this pathway is less well understood in T. brucei. Intriguingly, the recently completed T. brucei genome revealed that although this organism possesses a functional GLO2 [19], no apparent GLO1 gene or homologue could be identified [20]. This was unexpected, as the bloodstream form of T. brucei has an extremely high glycolytic flux and relies solely on substrate-level phosphorylation for ATP production [21]. Triose phosphates are a major source of methylglyoxal [12,13], and thus the reported antiproliferative effects of exogenous dihydroxyacetone [22] or endogenous modulation of triose phosphate isomerase in T. brucei [23] could be due to methylglyoxal toxicity. Should the absence of GLO1 from this pathogen be confirmed, it may have important implications for the viability of the glyoxalase system as a target for antitrypanosomatid chemotherapy. In this study, we attempted to further characterize the unusual methylglyoxal metabolism of T. brucei and directly compare it to that of T. cruzi and L. major.

Results and Discussion

Analysis of methylglyoxal-catabolizing enzymes in trypanosomatid cell extracts

Sequencing of the ‘Trityrp’ genomes has revealed several interesting distinctions between the cellular metabolism of T. brucei, T. cruzi and L. major [20]. In our current study, we sought to examine the apparent absence of a gene encoding a GLO1 homologue from the T. brucei genome, GLO1 being a ubiquitous enzyme required for the metabolism of methylglyoxal. Initially, the relative activities of enzymes involved in methylglyoxal metabolism were compared in these medically significant trypanosomatids. Whole cell extracts of T. cruzi epimastigotes, L. major promastigotes and T. brucei (bloodstream and procyclic forms) were prepared, and the activities of methylglyoxal-catabolizing enzymes were determined (Table 1). In keeping with previously published data [14,15], trypanothione-dependent GLO1 activity was detected in both L. major and T. cruzi extracts with specific activities of 85 and 42 nmol·min⁻¹·mg⁻¹, respectively. However, GLO1 activity could not be detected in extracts of T. brucei procyclic or bloodstream forms, with either trypanothione or glutathione hemithioacetals as substrate. In contrast, trypanothione-dependent GLO2 activity was detected in all cell lysates. With S-β-lactoyltrypanothione as a substrate, L. major extracts demonstrated GLO2 activity of 62.8 nmol·min⁻¹·mg⁻¹, over sixfold higher than that of T. cruzi extracts (8.8 nmol·min⁻¹·mg⁻¹). Despite the apparent lack of GLO1 activity, both T. brucei bloodstream form and procyclic extracts effectively metabolized S-β-lactoyltrypanothione, with specific activities of 18 and 23 nmol·min⁻¹·mg⁻¹, respectively. Trypanothione reductase activities were also assayed in each lysate to ensure adequate extraction of the parasites, and were in line with previously published data [24].

Western blot analyses of cell extracts

To confirm the absence of GLO1 from T. brucei at the protein level, immunoblots of trypanosomatid whole cell lysates were probed with L. major GLO1-specific polyclonal antiserum (Fig. 1). As expected, a protein of 16 kDa, which is equivalent to the predicted molec-
ular mass of GLO1, reacted strongly with the antiserum in both the *L. major* and the *T. cruzi* lysates. No GLO1-like protein was detected in whole cell lysates of *T. brucei* procyclics, despite overexposure of the blot. In combination with our enzymatic analysis of cell extracts, these data confirm the absence of a functional GLO1 enzyme within *T. brucei*. This situation is not entirely without precedence. Cestode and digenean parasitic helminths have been studied that lack GLO1 while maintaining high levels of GLO2 activity [25]. One explanation for the retention of this enzyme is that *T. brucei* GLO2 has methylglyoxal-independent functions. Indeed, human GLO2 has demonstrated substrate promiscuity in efficiently hydrolysing thiol esters of simple acids such as formic acid, succinic acid and mandelic acid [13]. The identification of the true physiological substrate of *T. brucei* GLO2 will form the basis of our future studies.

**Effects of methylglyoxal on trypanosomatid growth**

The absence of GLO1 from *T. brucei* suggested that these parasites may be particularly susceptible to the toxic effects of methylglyoxal. With this in mind, *T. cruzi*, *L. major* and *T. brucei* were grown in the presence of increasing methylglyoxal concentrations, and the relative growth of each culture was determined after 72 h (Fig. 2). To allow the direct comparison of the methylglyoxal sensitivity of these parasites, each cell line was adapted for growth in SDM-79 medium prior to analysis. *T. brucei* procyclics were the most sensitive to methylglyoxal toxicity, with an EC_{50} of 70 ± 2 μM, whereas *T. cruzi* epimastigotes and *L. major* promastigotes were 2.4-fold and 5.7-fold less sensitive, with EC_{50} values of 171 ± 11 and 397 ± 27 μM, respectively.

| Enzyme                          | *L. major* | *T. cruzi* | *T. brucei* procyclics | *T. brucei* bloodstream forms |
|---------------------------------|------------|------------|------------------------|-------------------------------|
| GLOI                            | 85.1 ± 3.8 | 42.3 ± 2.4 | < 5                    | < 5                           |
| GLOII                           | 62.8 ± 3.6 | 8.82 ± 0.29| 17.9 ± 2.1             | 22.9 ± 3.4                    |
| Methylglyoxal reductase         | 5.3 ± 0.7  | 4.8 ± 0.42 | 9.4 ± 1.1              | 10 ± 2.3                      |
| Lactaldehyde dehydrogenase      | 0.51 ± 0.004 | 0.48 ± 0.02* | 1.24 ± 0.11         | < 0.4                        |
| Trypanothione reductase         | 266 ± 30   | 133 ± 5.6  | 39.6 ± 2.8             | 46.3 ± 3.9                    |

* Activity measured in whole cell lysate.

Table 1. Analysis of methylglyoxal-catabolizing activities in trypanosomatid lysates. All enzymatic activities were assayed as described in Experimental procedures, and corrected for nonenzymatic background rates. Specific activities represent the means ± SD of six determinations from two independent experiments.

Fig. 1. Immunoblot analysis of trypanosomatid whole cell lysates. Immunoblots of whole cell extracts (30 μg of protein in each lane) from *T. cruzi* epimastigotes, *L. major* promastigotes and *T. brucei* procyclics were probed with antiserum to *L. major* GLO1.

Fig. 2. EC_{50} values for methylglyoxal against the ‘Tritryp’ trypanosomatids. The EC_{50} values for methylglyoxal against *L. major* promastigotes (open squares), *T. cruzi* epimastigotes (open triangles) and *T. brucei* procyclics (closed circles) were determined. The curves are the nonlinear fits of data using a two-parameter EC_{50}–0–100% equation provided by GRAFIT (see Experimental procedures). EC_{50} values of 70 ± 2 methylglyoxal, 171 ± 11 and 397 ± 27 μM were determined for *T. brucei*, *T. cruzi* and *L. major* with corresponding slope factors (m) of 3.0, 1.6 and 1.59, respectively. Data are the means of triplicate measurements.
Bloodstream trypanosomes could not be adapted for growth in SDM-79 medium, and attempts to determine the methylglyoxal sensitivity of these cells in HMI-9 medium proved unsuccessful, due to the propensity of methylglyoxal to react with thiols in this culture medium. In a previous study on the curative effect of methylglyoxal in cancer-bearing mice [26], Ghosh et al. established the pharmacokinetic properties of methylglyoxal in blood following oral dosing. Using this methodology, we examined the effects of methylglyoxal on an in vivo T. brucei infection. The maximum achievable methylglyoxal concentration in blood following oral dosing of mice was 20 µM, and at this level there was no discernible effect on the progression of the parasite infection (data not shown). These results suggest that the methylglyoxal EC₅₀ for bloodstream T. brucei in vivo is in excess of 20 µM.

**Trypanosomatid metabolism of methylglyoxal**

The rate of exogenous methylglyoxal metabolism by T. cruzi, L. major and T. brucei (bloodstream and procyclic forms) was determined (Fig. 3). Each cell line was resuspended in a minimal medium that had been preincubated with 1.5 mM methylglyoxal for 90 min. At defined intervals, culture supernatants were removed and analysed for residual methylglyoxal. In keeping with both our enzymatic analysis of whole cell lysates and EC₅₀ data, L. major promastigotes dealt with exogenous methylglyoxal most efficiently, with an initial rate of 67 nmol/min/mL. In comparison, T. cruzi epimastigotes were considerably less effective at metabolizing methylglyoxal (47.6 nmol/min/mL). However, T. brucei procyclics and bloodstream forms proved to be the least adept at dealing with this toxic oxoaldehyde, metabolizing methylglyoxal with initial rates of 7.4 nmol·min⁻¹·mL⁻¹ and 9.8 nmol·min⁻¹·mL⁻¹, respectively. These results suggest that although T. brucei is predicted to be the most vulnerable of the ‘Tritryp’ trypanosomatids to methylglyoxal toxicity, it can effectively metabolize methylglyoxal despite the absence of a complete glyoxalase pathway.

**Products of trypanosomatid metabolism of methylglyoxal**

In all studies to date, the principal product of thiol-dependent metabolism of methylglyoxal has been D-lactate [27–29]. Consequently, methylglyoxal-treated parasites were monitored for the production of lactate, using D-lactate and L-lactate dehydrogenase-based assays (Table 2). As expected, both L. major and T. cruzi cells produced considerable amounts of D-lactate following exposure to methylglyoxal, accounting for approximately 30% of free methylglyoxal in the medium. In contrast, T. brucei (procyclics and bloodstream forms) produced only trace amounts of D-lactate. Instead, methylglyoxal-treated T. brucei procyclics and bloodstream forms produced significant quantities of the stereoisomer L-lactate (120 and 221 µM in 2 h, respectively). The sixfold higher rate of L-lactate production by bloodstream parasites in the absence of exogenous methylglyoxal reflects the extremely high glycolytic rate in this developmental form of the African trypanosome [30]. The addition of methylglyoxal marginally decreased the amount of L-lactate detected in the supernatants of both L. major and T. cruzi cultures. These data suggest that T. brucei may metabolize methylglyoxal by an alternative pathway.

In a previous study [31], Ghoshal et al. identified NADPH-dependent methylglyoxal reductase activity in *Leishmania donovani* promastigotes. These parasites were shown to metabolize approximately 1.2% of the exogenous methylglyoxal added to cultures via this
logues of these enzymes were identified in the genome, and neither NAD+-dependent nor NADP+-dependent methylglyoxal dehydrogenase activities were detected in T. brucei extracts (data not shown). This protein was not evident in cells transfected with an unrelated vector (pLew100–luciferase).

To complete the metabolism of l-lactaldehyde to lactate, T. brucei would require a functional l-lactaldehyde dehydrogenase. Although l-lactaldehyde dehydrogenase activity has previously been detected in L. donovani cell lysates [31], it has yet to be identified in either T. cruzi or in T. brucei. Using l-lactaldehyde as a substrate, l-lactaldehyde dehydrogenase activity was measured in all three insect-stage trypanosomatid cell lysates (Table 1), and was found to be relatively similar in L. major and T. cruzi lysates (0.51 ± 0.004 and 0.48 ± 0.02 nmol·min⁻¹·mg⁻¹, respectively). In comparison, l-lactaldehyde dehydrogenase activity was found to be elevated approximately 2.4-fold in T. brucei procyclic cell lysates (1.24 ± 0.11 nmol·min⁻¹·mg⁻¹). However, activity could not be detected in the bloodstream stage of the parasite. These studies confirm that procyclic T. brucei organisms are capable of metabolizing methylglyoxal, via a methylglyoxal reductase-dependent pathway, to L-lactate; however, it remains to be seen whether this is the predominant pathway for methylglyoxal detoxification in these cells.

Our failure to detect NAD⁺-dependent l-lactaldehyde dehydrogenase activity in T. brucei bloodstream forms may be due to technical reasons, such as NADH oxidation via the glycerophosphate oxidase system masking the formation of NADH.

Expression of T. cruzi GLO1 (TcGLO1) in T. brucei

Can T. brucei utilize a complete glyoxalase system? To address this question, a tetracycline-inducible pLew100–TcGLO1 construct was generated and transfected into both bloodstream and procyclic cells. Western blot analysis of transgenic parasites, following induction with tetracycline, confirmed the expression of a 16-kDa protein that reacted strongly with GLO1-specific antiserum (Fig. 4; bloodstream data not shown). This protein was not evident in cells transfected with an unrelated vector (pLew100–luciferase). Antiserum against T. brucei pteridine reductase I was used to establish equal loading of samples. The expression of recombinant TcGLO1 in procyclics and bloodstream forms was confirmed when GLO1 activity (23.0 ± 1.9 and 38.2 ± 1.9 nmol·min⁻¹·mg⁻¹, respectively) was detected in cell extracts. Indeed, the rate of exogenous methylglyoxal metabolism in these transgenic T. brucei cell lines increased markedly, with GLO1-expressing procyclic and bloodstream cells metabolizing the toxic oxoaldehyde 1.7-fold and

Table 2: Comparison of methylglyoxal-stimulated D-lactate and L-lactate production by trypanosomatids. Parasites were incubated with or without methylglyoxal for 2 h prior to analysis. Data represent the mean ± SD of triplicate determinations. See Experimental procedures for further details.

| Organism       | D-Lactate | L-Lactate |
|----------------|-----------|-----------|
|                | Plus methylglyoxal | Minus methylglyoxal | Net |
| L. major       | 398 ± 9 | 49 ± 9 | 337 | 7 ± 0.6 | 12 ± 0.1 | –5 |
| T. cruzi       | 303 ± 3 | 8 ± 0.8 | 295 | 9 ± 0.3 | 13 ± 0.1 | –4 |
| T. brucei procyclics | 18 ± 0.2 | 8 ± 0.1 | 11 | 141 ± 5 | 21 ± 0.1 | 120 |
| T. brucei bloodstream forms | 68 ± 9 | 50 ± 2 | 18 | 355 ± 42 | 134 ± 18 | 221 |
2.7-fold more effectively, respectively (Table 3). Most importantly, TcGLO1-expressing T. brucei procyclics were almost 3.5-fold less sensitive to methylglyoxal than wild-type or luciferase-expressing cells.

To confirm that enhanced methylglyoxal tolerance in GLO1-expressing T. brucei was due to complementation of the glyoxalase system, lactate production in the supernatants of methylglyoxal-treated wild-type and transgenic cells was measured (Table 4). Whereas l-lactate levels in the supernatants of GLO1-expressing T. brucei (bloodstream forms and procyclics) were very similar to those of the wild-type, d-lactate production was found to be significantly higher (~3-fold, \( P < 0.0001 \)). d-Lactate levels failed to reach those seen in the supernatants of methylglyoxal-treated L. major and T. cruzi, but were sufficient to suggest that GLO1 expression in T. brucei procyclic and bloodstream parasites results in a complete glyoxalase system.

### Table 3. Comparison of GLO1 activity, methylglyoxal sensitivity and methylglyoxal metabolism in T. brucei wild-type and transgenic cell lines. ND, not determined.

| Cell line            | GLO1 activity (nmol·min\(^{-1}·mg\(^{-1}\)) | EC\(_{50}\) (\(\mu\)M) | Methylglyoxal metabolized\(^a\) (nmol·mL\(^{-1}·h\(^{-1}\)) | 
|----------------------|---------------------------------------------|------------------------|------------------------------------------------------|
| T. brucei            |                                             |                        |                                                      |
| Procyclics           | < 5                                         | 53.4 ± 2.9             | 246 ± 21                                             |
| Bloodstream forms    | < 5                                         | ND                     | 300 ± 32                                             |
| pLew100–luciferase\(^b\) |                                             |                        |                                                      |
| Procyclics           | < 5                                         | 47.8 ± 3.8             | 197 ± 16                                             |
| Bloodstream forms    | < 5                                         | ND                     | 260 ± 28                                             |
| pLew100–TcGLO1\(^c\) |                                             |                        |                                                      |
| Procyclics           | 23.0 ± 1.9                                  | 175 ± 5.6\(^d\)       | 387 ± 27                                             |
| Bloodstream forms    | 38.2 ± 1.9                                  | ND                     | 810 ± 40                                             |

\(^a\) Values are the weighted means of three independent experiments. \(^b\) All data represent the mean ± SD of six determinations from two independent experiments. \(^c\) Cell lines were grown in the presence of tetracycline for 24 h prior to analysis. \(^d\) \( P < 0.001 \) as compared to T. brucei.

### Table 4. Comparison of methylglyoxal-stimulated d-lactate and l-lactate production by wild-type and transgenic T. brucei cell lines. Data represents the mean ± SD of six determinations from two independent experiments.

| Cell line            | d-Lactate | l-lactate | Net | d-Lactate | l-lactate | Net |
|----------------------|-----------|-----------|-----|-----------|-----------|-----|
|                       | Plus      | Minus     |     | Plus      | Minus     |     |
| T. brucei            |           |           |     |           |           |     |
| Procyclics           | 22 ± 3    | 10 ± 2    | 12  | 148 ± 7   | 24 ± 3    | 124 |
| Bloodstream forms    | 68 ± 9    | 50 ± 2    | 18  | 355 ± 42  | 134 ± 18  | 221 |
| pLew100–luciferase\(^b\) |           |           |     |           |           |     |
| Procyclics           | 17 ± 2    | 9 ± 1     | 8   | 134 ± 12  | 19 ± 2    | 115 |
| Bloodstream forms    | 59 ± 2    | 48 ± 3    | 11  | 329 ± 26  | 118 ± 21  | 211 |
| pLew100–TcGLO1\(^c\) |           |           |     |           |           |     |
| Procyclics           | 57 ± 4\(^a\) | 19 ± 2    | 38  | 122 ± 9   | 22 ± 2    | 100 |
| Bloodstream forms    | 183 ± 24\(^a\) | 71 ± 5    | 112 | 308 ± 14  | 105 ± 8   | 203 |

\(^a\) \( P < 0.001 \) as compared to T. brucei.

### Implications for parasite chemotherapy

Mammalian cells maintain a repertoire of four pathways for metabolism of methylglyoxal [33], whereas our studies suggest that the African trypanosome may be solely dependent upon methylglyoxal reductase (Fig. 5). The absence of a functioning glyoxalase system within T. brucei, recognized as the principal route of oxoaldehyde detoxification in almost all cells, is especially perplexing. As methylglyoxal is generated primarily as a byproduct of glycolysis, and African trypanosomes are entirely dependent upon glycolysis for energy, it would be reasonable to assume that T. brucei would preserve robust methylglyoxal-metabolizing systems. Without an...
intact glyoxalase pathway, these cells should be particularly vulnerable to methylglyoxal toxicity, and our current studies appear to confirm this. These findings have broad implications for the targeting of methylglyoxal metabolism for antitrypanosomatid chemotherapy. Previous studies have suggested that the contrasting substrate specificities of the human and trypanosomatid glyoxalase enzymes (GLO1 and GLO2) make them attractive targets for rational drug design [14,15,19]. Whereas this may still be the case in *T. cruzi* and *Leishmania* spp., methylglyoxal reductase is clearly a more promising drug target in the African trypanosome. Identification of the genes that encode this enzyme in *T. brucei* should now be a priority.

**Experimental procedures**

**Cell lines and culture conditions**

*L. major* promastigotes (Friedlin strain; WHO designation MHOM/JL/81/Friedlin), procyclic trypomastigotes of *T. brucei brucei* S427 29-13 and epimastigotes of *T. cruzi* CL Brener (genome project standard clone) were adapted for growth in SDM-79 medium supplemented with 10% fetal bovine serum (Gibco, Paisley, UK) and haemin (100 mg L\(^{-1}\)). *L. major* promastigotes were grown at 24 °C with shaking, and *T. brucei* and *T. cruzi* were cultured at 28 °C. *T. brucei* bloodstream forms were cultured at 37 °C in modified HM19 medium (56 μM 1-thioglycerol was substituted for 200 μM 2-mercaptoethanol) supplemented with 2.5 μM L-G418 to maintain expression of T7 RNA polymerase and the tetracycline repressor protein [34].

In order to directly compare the effects of methylglyoxal on the growth of these trypanosomatids, triplicate cultures containing methylglyoxal were seeded at 5 \( \times \) \( 10^5 \) parasites per mL. As methylglyoxal interferes with the Alamar blue assay for viable cells, cell densities were determined using the CASY Model TT cell counter (Schaefr, Renningen, Germany) after culture for 72 h. Concentrations of inhibitor causing a 50% reduction in growth (EC\(_{50}\)) were determined using the following two-parameter equation by nonlinear regression using GRAFIT:

\[
y = \frac{100}{1 + ([I]/EC_{50})^m}
\]

where the experimental data were corrected for background cell density and expressed as percentages of the uninhibited control cell density. In this equation, [I] represents inhibitor concentration, and \( m \) is the slope factor.

**Analysis of methylglyoxal-catabolizing enzymes in trypanosomatid cell lysates**

*L. major* promastigotes (2 \( \times \) \( 10^7 \) mL\(^{-1}\), 1 L), *T. cruzi* epimastigotes (3 \( \times \) \( 10^7 \) mL\(^{-1}\), 1 L) and *T. brucei brucei* procyclics (2 \( \times \) \( 10^7 \) mL\(^{-1}\), 1 L) were pelleted by centrifugation (1600 g, 10 min, 4 °C), washed twice in 20 mM Tris (pH 7.0) containing 0.1 mM sucrose, and resuspended in cell lysis buffer (10 mM potassium phosphate, pH 7.0). For biological safety, parasites were inactivated by three cycles of freezing and thawing, before lysis under pressure (30 kpsi) using a one-shot cell disruptor (Constant Systems, Daventry, UK). *T. brucei* bloodstream forms (4 \( \times \) \( 10^9 \) cells), harvested from rats as previously described [35], were lysed using an alternative method. Cells were pelleted by centrifugation...
(800 g, 10 min, 4 °C), washed once in PSG buffer [NaCl/Pi, pH 8.0, 1.5% (w/v) glucose and 0.5 mgmL⁻¹ BSA], resuspended in ice-cold de-ionized dH2O, (500 µL) and vortexed. Lysed bloodstream trypanosomes were then incubated on ice for 10 min prior to the addition of 2× lysis buffer (500 µL) and further vortexing. From this point, all lysates were treated in an identical manner. Following centrifugation (800 g, 20 min, 4 °C), cell supernatants were collected and dialysed against 50 mM Hepes (pH 7.0) with 25 mM NaCl and 150 µM 2-mercaptoethanol at 4 °C to remove components of less than 3.5 kDa. The protein concentration of each lysate was determined using Bradford reagent (Bio-Rad, Hemel Hempstead, UK). GLO1 activity in the trypanosomatid cell lysates was measured by monitoring the formation of S-d-lactoyltrypanothione spectrophotometrically at 240 nm [14]. Trypanothione and methylglyoxal were preincubated at 25 °C for 10 min in 50 mM Hepes (pH 7.0) plus 25 mM NaCl and 50 µM adduct, and 100 µM free thiol. Reactions were initiated with enzyme extract. Methylglyoxal reductase and GLO2 activities were determined as previously described [14,31,36]. The activity of trypanothione reductase, used as a control enzyme, was assayed as previously described [37].

L-Lactaldehyde dehydrogenase activity in trypanosomatids

L-Lactaldehyde, the substrate of l-lactaldehyde dehydrogenase, was prepared from d-threonine, as previously described [38]. Briefly, 25 mmol of d-threonine, 9.1 g of ninhydrin and 600 mL of 0.05 M sodium citrate buffer (pH 5.4) were combined and boiled for 15 min with continual stirring. After being cooled to room temperature, the mixture was filtered and treated with sufficient Dowex 1-X8 resin (bicarbonate form) to raise the pH to 6.5. After stirring for a further 2–3 h, the resin was again filtered, and the filtrate was adjusted to pH 4.0 by the addition of Dowex 50 resin (hydrogen ion form). Following filtration, the filtrate was concentrated down to 50–100 mL using a rotary evaporator. The resulting concentrate was then sequentially treated with concentrated HCl (pH 7.0) plus 25 mM NaCl, 50 µM adduct, and 100 µM free thiol. Reactions were initiated with enzyme extract. Methylglyoxal reductase and GLO2 activities were determined as previously described [14,31,36]. The activity of trypanothione reductase, used as a control enzyme, was assayed as previously described [37].

Western blot analyses of trypanosomatid cell extracts

Polyclonal antisera against L. major GLO1 were raised in adult male Wistar rats. An initial injection of 100 µg of purified antigen, emulsified in complete Freund’s adjuvant, was followed by two identical booster injections of antigen emulsified in Freund’s incomplete adjuvant at 2 week intervals.

Trypanosomatid whole cell extracts (30 µg) were separated by SDS/PAGE and subsequently transferred onto nitrocellulose. After blocking with 7% skimmed milk in NaCl/P, for 1 h, blots were incubated with L. major GLO1 polyclonal antiserum (1:700 dilution) for 1 h, washed in NaCl/P, containing 0.1% (v/v) Tween-20, and then incubated with a secondary antibody [rabbit anti-(rat IgG)] (Dako, Ely, UK; 1:10 000 dilution). Immunoblots were developed using the ECL plus (enhanced chemiluminescence) system from Amersham Biosciences (Piscataway, NJ, USA).

Analysis of methylglyoxal metabolism in trypanosomatids

Mid-log L. major promastigotes, T. cruzi epimastigotes and T. brucei procycls (4 × 10⁸ cells) were pelleted by centrifugation (1600 g, 10 min, 4 °C) and washed in a maintenance medium (250 mM sucrose, 25 mM Tris, pH 7.4, 1 mM EDTA, 8 g L⁻¹ glucose, and 0.5 mgmL⁻¹ BSA). Cells were then resuspended at 1 × 10⁶ mL⁻¹ in...
medium supplemented with 2.5 μg·mL⁻¹ G418, to maintain expression of T7 RNA polymerase and the tetracycline repressor protein, and 2.5 μg·mL⁻¹ phleomycin. Methylglyoxal metabolism in the transfected cell lines was analysed, as previously described, following induction of recombinant protein expression with tetracycline (2 μg·mL⁻¹, 24 h).

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