Journal of Enzyme Inhibition and Medicinal Chemistry, February 2004 Vol. 19 (1), pp. 57–63

Trypanothione Reductase Activity is Prominent in Metacyclic Promastigotes and Axenic Amastigotes of *Leishmania amazonesis*. Evaluation of its Potential as a Therapeutic Target

DENISE B. CASTRO-PINTO[a], AUREA ECHEVARRIA[b], MARCELO S. GENESTRA[a], LÉA CYSNE-FINKELSTEIN[a] and LEONOR L. LEON[a,*]

[a]*Department of Immunology, Oswaldo Cruz Institute, FIOCRUZ, Av. Brasil, 4365-Manguinhos CEP 21042-900, Rio de Janeiro/RJ, Brazil;* [b]*Department of Chemistry, UFRRJ, Brazil*

(Received 8 June 2003; In final form 17 August 2003)

The activity of trypanothione reductase in *Leishmania amazonensis* was evaluated and it was demonstrated that TR is expressed in the soluble fractions of infective promastigotes and amastigotes, while non-infective promastigotes expressed the enzyme at basal levels. This data allows an association of enzyme activity and the infective capacity of the parasite. We have also previously demonstrated that amidine compounds (N, N′-diphenyl-4-methoxy-benzamidine and pentamidine) were active against this parasite. Here, experiments concerning the effect of these compounds on TR activity, showed that both compounds significantly inhibited the enzyme. However, against glutathione reductase, only pentamidine showed a significant inhibitory action, suggesting an association with the toxic effects of this drug used in the clinic for the treatment of leishmaniasis.

Keywords: Trypanothione reductase; Amastigotes; Promastigotes; Infectivity; Drug target; *Leishmania amazonensis*

INTRODUCTION

Leishmania parasites are associated with different clinical forms of a disease referred to as leishmaniasis. During *Leishmania* life cycle the microbiocidal interactions between parasite and host cells occur in two stages, which includes (a) the initial phagocytosis of promastigotes by macrophages with a consequent oxidative response, stimulated by the phagocytosis event and (b) the macrophage activation when recently differentiated amastigotes initiate the infection. Efficient evasion from toxic microbiocidal molecules produced at each stage of infection is important for *Leishmania* to be able to initiate and maintain host cell infection. As a consequence of the increase of the respiratory burst, parasites are exposed to toxic oxygenated intermediates, such as, the anion super oxide ($O_2^{-\cdot}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($OH^{-\cdot}$). Trypanosomatids, as the majority of cells are, able to avoid the toxic effect of $O_2^{-\cdot}$, transforming it into H_2O_2 through a super oxide dismutase (SOD) enzyme.

Promastigotes undergo a developmental change as they progress from the logarithmic to stationary phase (presence of metacyclic forms) in culture, which is accompanied by an increase in resistance to H_2O_2 induced injury and an increase in virulence.[1] Trypanosomatids have been reported to be deficient in their ability to metabolize H_2O_2 and be particularly sensitive to H_2O_2 oxidant stress due to their lack of glutathione reductase (GR) and catalase.[2] However, it has been demonstrated that trypanosomatids have a very efficient system for maintaining a reducing intracellular environment, consisting of trypanothione, a covalent conjugate of glutathione and spermidine [^{1}N, ^{8}N-bis (glutathionyl) spermidine; T $(S)_2$] and trypanothione reductase (TR).[3,4] TR has been identified in all trypanosomatids species studied up to the present time such as *Crithidia fasciculata*,[5,6] *Trypanosoma cruzi*,[7–9] *T. brucei*,[10] *Leishmania donovani*[11] and *L. tarentolae*.[12]

GR and TR which belong to the same oxidoreductase group, however, present well defined specificity concerning their substrates, which indicates that TR is a good target for trypanocidal drugs. Several

*Corresponding author. Tel.: + 55-21-38658131. Fax: + 55-21-22900479. E-mail: lleon@ioc.fiocruz.br

ISSN 1475-6366 print/ISSN 1475-6374 online © 2004 Taylor & Francis Ltd
DOI: 10.1080/14756360310001624966

compounds have been described as selective inhibitors of TR, showing a weak interaction with GR.[13–17]

It is interesting to notice that there is no reference in all the studies cited in the literature concerning a comparative analysis of TR and different parasite evolutive stages.

Our group has shown in previous work, that N, N′-diphenyl-4-methoxy-benzamidine, was highly effective against *L. amazonensis* and considering that TR has become a very interesting target for trypanocidal drugs, we decided to evaluate the effect of this compound on TR activity. Consequently, here the activity of TR was evaluated in infective promastigotes (containing a high amount of metacyclic forms), non-infective promastigotes (no metacyclic forms detected) and amastigotes of *L. amazonensis*. The results showed that TR activity was prominent in both promastigotes with a high percentage of metacyclic forms (about 73%) and amastigotes, suggesting an association between the enzyme activity and the parasite infectivity. The effect of amidine derivatives (methoxy-derivative and pentamidine) was also evaluated in both TR (from parasites) and GR activities (from a macrophage cell line). It was observed that both compounds were able to inhibit TR to the same level. However, only pentamidine showed a significant inhibition of GR, which could be associated with its toxicity already observed for the treatment of leishmaniasis in the clinic, where it is used as a second line treatment for the disease.

MATERIALS AND METHODS

Parasite

Leishmania amazonensis (MHOM/BR/77/LTB0016 strain) promastigotes [infectives (IP)/non-infectives (NIP)], axenic and lesion amastigotes (AA/LA) were used. Promastigotes were obtained from Balb/c mice lesions, inoculated in biphasic medium consisting of NNN (Novy, McNeal & Nicole), with 15% agar and defibrinated blood rabbit as the solid phase and Schneider's medium, pH 7.2, as liquid phase, supplemented with inactivated fetal calf serum (10%) (FCS), 1 mmol/L L-glutamine, 100 UI penicillin G and 100 μg/mL of streptomycin. Promastigotes cultures were incubated at 26°C.

Lesion amastigotes were obtained from infected Balb/c mice. Lesions were homogenized and centrifuged at 1000 × g for 5 min at 22°C. The supernatant was centrifuged again at 2000 × g for 5 min at 22°C, the final pellet containing the amastigotes. Axenic amastigotes were obtained following the protocol described by Cysne-Finkelstein and collaborators.[18] Briefly, amastigotes isolated as described above were cultivated in Schneider's medium containing 1 mol/L HEPES buffer. After culture for three days, the recently transformed promastigotes with a high proportion of metacyclic forms were incubated at 32°C and pH 5.5, also in Schneider's medium, but with 20% FCS and a lower antibiotic concentration (60 UI penicillin and 60 μg/mL streptomycin). Axenic amastigotes were maintained in culture by passages every 10 days.

Cell Line

The macrophage lineage J774-G8 was utilized, which was grown in RPMI medium, pH7.0, enriched with 10% FCS, 10U/mL of a mixture of penicillin and streptomycin, 2 g HEPES and 2 g $NaHCO_3$. After 72 h of incubation at 37°C in a 5% CO_2 atmosphere, cells were harvested from the medium and prepared for the enzyme assay.

Cellular Fractionation

The samples used for the enzyme assay were the following: soluble fraction (SF) and an enriched membrane fraction (MF) of infective promastigotes (up to 5 passages in culture); SF of non-infective promastigotes (more than 100 passages in culture); SFs of axenic and lesion amastigotes; SF of J774-G8 cells.

All cells were harvested from the medium and centrifuged twice in order to remove cellular debris. The final pellet was resuspended in 40 mmol/L HEPES buffer and 1 mmol/L EDTA, lysed in a Dounce homogenizer, centrifuged at 12,500 × g for 15 min and the supernatant considered as the soluble fraction (SF).[19] In the case of infective promastigotes, the pellet was solubilized in 1% Triton X-100,[20] sonicated, centrifuged at 1500 rpm for10 min and the supernatant considered as the enriched membrane fractions (MF). The protein content (mg/mL) of all samples was measured according to Johnstone and Thorpe.[21]

Synthesis of the Compound

N, N′-diphenyl-4-methoxy-benzamidine was synthesized from 4-methoxy-benzanilide with aniline and phosphorus pentachloride (PCl_5) and the mixture refluxed for 2 h. The product was purified by recrystalization from methanol or toluene and fully characterized by IR, ^1H and ^{13}C NMR spectroscopy and mass spectrometry.[22] Pentamidine was from a commercial source (FH Faulding & Co Limited, Australia). Figure 1 shows the chemical structures of the compounds used.

TR Activity Assay

TR activity was detected spectrophotometrically by measuring NADPH consumption at 340 nm.[15] The assay mixture contained 40 mmol/L HEPES, pH 7.5,

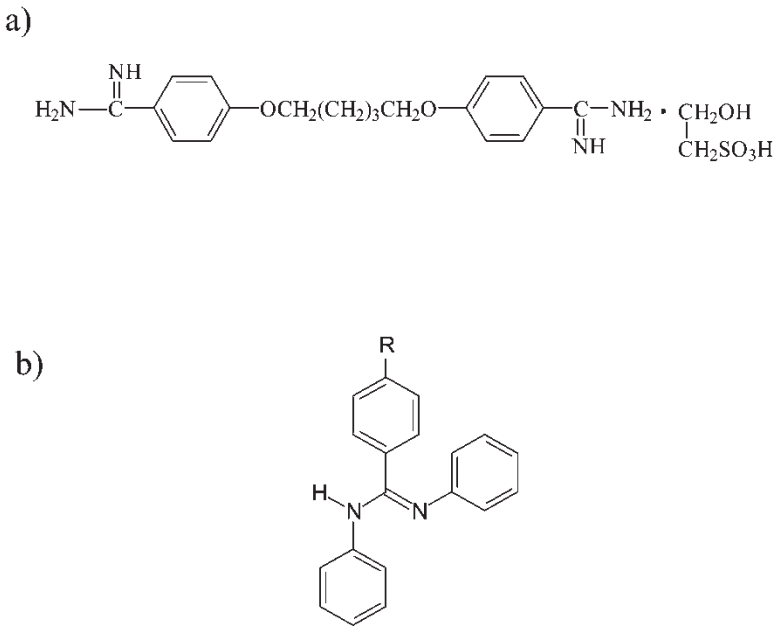

FIGURE 1 Chemical structure of amidines: a) pentamidine isethionate (reference compound); b) N, N′-diphenyl-4-(R)-benzamidine, where R = OCH_3 (test compound).

1 mmol/L EDTA, 100 μmol/L NADPH and the parasite sample (1 mg/mL of protein). Controls were run without NADPH. NADPH consumption was followed for up to 60 min, with 10 min intervals for all samples. To ensure that NADPH consumption was associated with TR activity, an assay was carried out where an excess (500 μmol/L and 1000 μmol/L) of oxidized glutathione (GSSG) was added, as described by Krauth-Siegel *et al*.[23] The reduced trypanothione T $(SH)_2$ formed reacts with the excess GSSG to produce more oxidized trypanothione $T(S)_2$, so increasing NADPH consumption according to the Scheme:

$$T(S)_2 + NADPH + H^+ \rightarrow T(SH)_2 + NADP \text{ (through TR action)} \quad (1)$$

$$T(SH)_2 + GSSG(\text{excess}) \rightarrow T(S)_2 + GSH \text{ (non–enzymatic reaction)} \quad (2)$$

TR/GR Activity Assay in the Presence of Amidine Derivatives

To evaluate the effect of amidine compounds on TR activity in *L. amazonensis*, the infective promastigote soluble fraction was used (1 mg/mL protein). The enzyme assay was carried out as described above and the amidine compounds were added to the reaction mixture in concentrations equivalent to their IC_{50} values over a 24 h period. The IC_{50} values were 14 μmol/L and 0.46 μmol/L, for the methoxy-amidine and pentamidine, respectively. In order to evaluate the effect of the amidine compounds on GR activity, the soluble fraction of macrophage cells (1 mg/mL of protein) was used, as described for TR.

Statistical Analysis

Significance was determined using a non-paired *t*-Student test. Differences were considered to be significant when $p < 0.05$.

RESULTS

TR Activity of *L. amazonensis* Promastigotes

The TR activity, measured as NADPH consumption, did not vary at all assay time intervals in the SF of both infective and non-infective promastigotes, as well in the MF of infective promastigotes ($p > 0.05$). However, in a comparative analysis between NADPH consumption by the SFs of the non-infective and infective promastigotes, a higher consumption was observed by the latter, suggesting a high TR activity. The difference between the TR activity in these two parasite forms was highly significant ($p < 0.05$) (Fig. 2). Furthermore, the TR activity of the infective promastigotes MF, was even less than that for the non-infective ones (data not shown).

TR Activity of *L. amazonensis* Amastigotes

The evaluation of the TR activity of both amastigote forms (lesion and axenic) SF, showed that in this case enzyme activity was much higher than that observed for the infective promastigotes ($p < 0.05$). An alteration in NADPH consumption was not

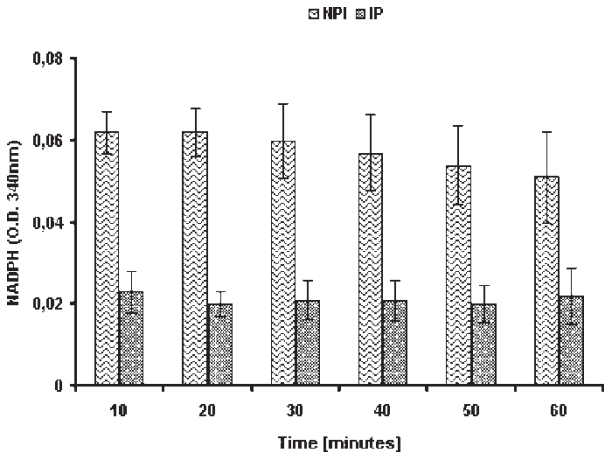

FIGURE 2 NADPH consumption associated with trypanothione reductase (TR) activity of promastigotes soluble fractions (SFs) (infectives/non-infectives) of *L. amazonensis*. The co-factor consumption was evaluated over 60 min at intervals of 10 min as described in Methods.

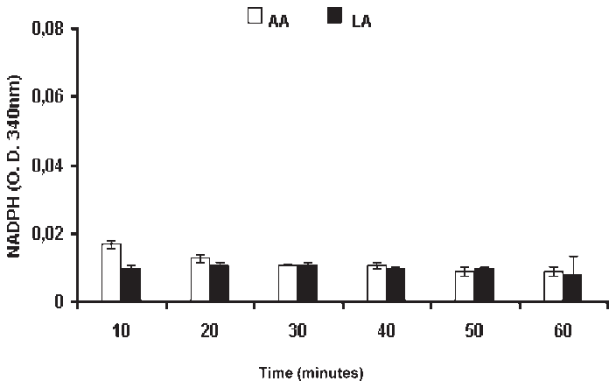

FIGURE 3 NADPH consumption associated with trypanothione reductase (TR) activity of lesion and axenic amastigotes soluble fractions (SF) of *L. amazonensis*. The co-factor consumption was evaluated over 60 min at intervals of 10 min as described in Methods.

observed in both forms during the whole period of the experiment ($p > 0.05$). It was not possible to explain the significant difference observed in the initial 10 min reading for NADPH consumption between the two forms, being higher for the lesion amastigote ($p < 0.05$) (Fig. 3).

TR Activity in the Presence of the Amidine Derivatives

Previous work from our laboratory showed that N,N′-diphenyl-4-(R)-benzamidine derivatives were active against *L. amazonensis* promastigotes and axenic amastigotes, the most effective being the methoxy-derivative.[24,25] The effect of this compound was now evaluated on the TR activity of the soluble fraction of promastigotes, using pentamidine as reference drug. It was observed that both compounds significantly inhibited enzyme activity ($p < 0.05$), compared to the controls without drugs, as measured by NADPH consumption. Also evaluated was the effect on the redox system of the host (GSSR/GR), which is an analog to the parasite redox system [$T(S)_2$/TR], where it was observed that while the methoxylated derivative had no effect on the GR from the host system ($p > 0.05$), pentamidine was able to decrease significantly this activity ($p < 0.05$) (Figs. 4 and 5).

DISCUSSION

During its infective cycle in the vertebrate host, *Leishmania* parasites must survive the hazardous

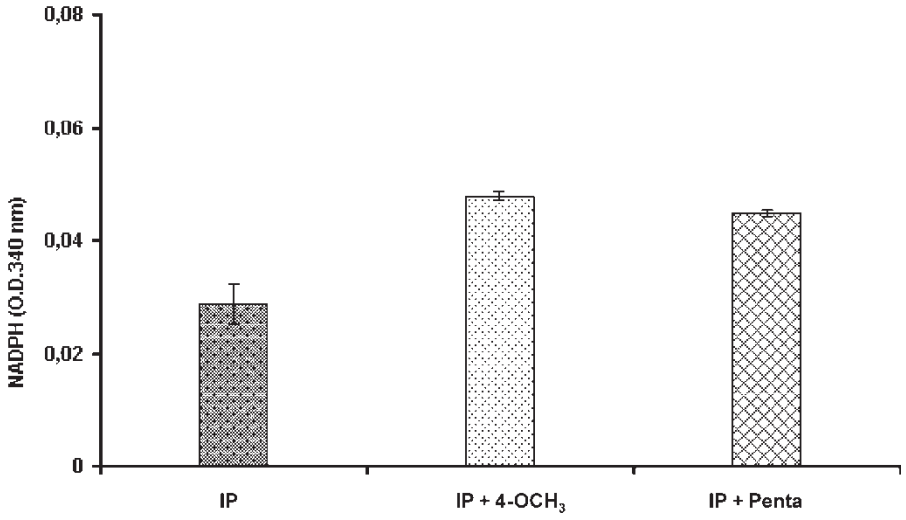

FIGURE 4 Effect of amidine derivatives on trypanothione reductase (TR) activity of SFs of infective promastigotes (IP) of *L. amazonensis*. The enzyme activity was evaluated through NADPH consumption as described in Methods. 4-OCH_3 = 4-methoxy-amidine. Penta = pentamidine.

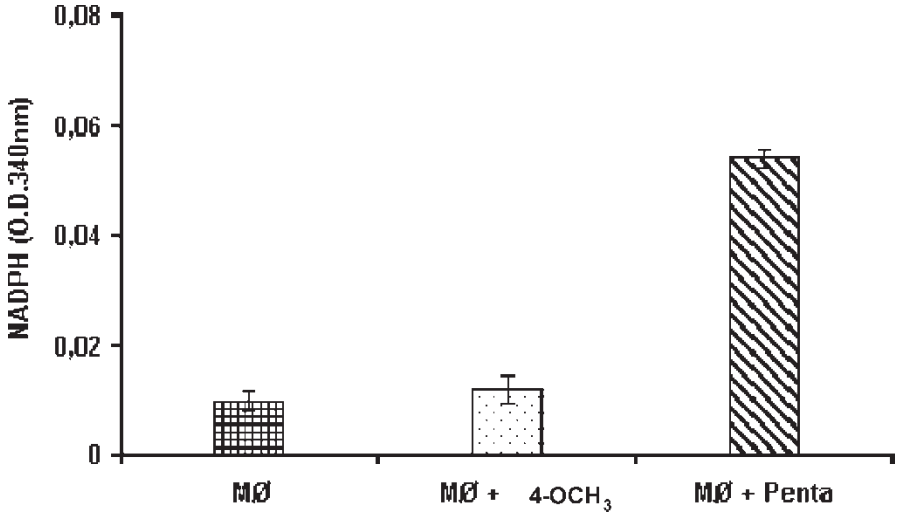

FIGURE 5 Effect of amidine derivatives on glutathione reductase (GR) activity of SFs of J774-G8 macrophage cell line. The enzyme activity was evaluated through NADPH consumption as described in Methods. 4-OCH_3 = 4-methoxy-amidine. Penta = pentamidine. M∅ = macrophage.

environment of the macrophage. Trypanosomatids maintain their intracellular redox balance through mechanisms that are completely different from those of the mammalian hosts. These parasites do not have glutathione reductase (GR, EC 1.6.4.2), which is the main enzyme to maintain the intracellular redox environment in most organisms. Instead, they have TR (TR, EC 1.6.4.8), an enzyme responsible for maintaining trypanothione in its reduced form, this being the central molecule in the redox defense system of trypanosomatids. TR and GR are members of the NADPH-dependent flavoprotein oxidoreductases family and are structurally related.[4,5,7,10]

The X-ray crystal structure of the enzyme TR, isolated from the trypanosomatid organism *Crithidia fasciculata*, has been solved by molecular replacement. The search model was the crystal structure of human GR that shares approximately 40% sequence identity with TR. The trypanosomatid enzyme assumes a similar biological function to GR but it has an enlarged active site and a number of amino acid differences, steric and electrostatic, which allows it to process only the unique substrate trypanothione and not glutathione. This protein represents a prime target for the chemotherapy of several debilitating tropical diseases caused by protozoan parasites belonging to the genera *Trypanosoma* and *Leishmania*. The structural differences between the parasite and host enzymes and their substrates thus provide a rational basis for the design of new drugs active against trypanosomes.[26]

It has been show that the TR genes of *L. donovani* and *L. major*[27] and *Trypanosoma brucei*,[28] are required for parasite intracellular survival and exhibit increased sensitivity to oxidative stress. Also, the disruption of TR genes in species of *Leishmania* from the Old World decreases its ability to survive within the macrophages.[27–29] The present data corroborated these results, since it was observed that TR activity is directly associated to the infectivity of *L. amazonensis*. It has been expected that promastigotes with a high percentage of metacyclic forms (73%) would have a system to allow them to initiate the infection and amastigotes must be more efficient to keep the development of the parasite within the so-called hostile macrophage environment.

The glutathione redox system has been described as protecting the mitochondrion from oxidant damage. Considering that the glutathione system is essential for mitochondrial functions, it would be expected that the trypanothione system would have a similar distribution pattern.[4] However, it was not observed in African trypanosomes, where trypanothione/TR is exclusively localized in the cytosolic compartment. Our results showed that the enriched membrane fraction of *L. amazonensis* infective promastigotes showed no TR activity by considering the low NADPH consumption. It has been suggested that either glutathione or trypanothione, would be transferred from the cytosol to mitochondria, where they remove free radicals in a non-enzymatic way.[30]

New drugs against these parasitic protozoa are urgently needed since currently available chemotherapy is not at all satisfactory. One promising approach towards the development of new drugs is based on the design of specific enzyme inhibitors. Trypanosomes and *Leishmania* possess a unique thiol metabolism in which the ubiquitous glutathione/GR system is replaced by trypanothione and TR. The dithiol trypanothione is the key molecule for the synthesis of DNA precursors, ascorbate homeostasis, the detoxification of hydroperoxides and the sequestration/export of thiol conjugates. The uniqueness of trypanothione makes the function

of this molecule an attractive target in antileishmanial drug design.[31] Attempts to exploit TR as a chemotherapeutic target lead to the design of competitive and irreversible inhibitors of the enzyme.

Among the several amidine derivatives[22] tested by our group on *L. amazonensis*, it was found that the most effective compound possessed a 4-methoxy group substituent.[24,25] The drug showed a high effectiveness against *Trypanosoma evansi* trypomastigotes, *T. cruzi* and PKA-*L. amazonensis* activity.[24,32,33] Thus, in order to verify the effect of the 4-methoxy-amidine on TR-*L. amazonensis*, an assay was carried out to see if a direct action exists by this compound on NADPH consumption by TR, where the mixture reaction contained all the components involved in TR reaction. The results showed that this compound was able to inhibit significantly the TR activity of the *L. amazonensis* promastigotes soluble fraction as seen by less consumption of NADPH. This data suggests that 4-methoxy-amidine interferes with TR activity in *L. amazonensis*, by an unknown mechanism. The literature suggests a potential mechanism of action for pentamidine by interaction with kDNA, not an intercalation, but a ligation on the kDNA minor grooves.[34] Experiments with pentamidine and 4-methoxy-amidine, showed by electrophoresis, that while pentamidine interacts with kDNA minor grooves, the 4-methoxy-amidine did not give any change in the electrophoretic profile.[35] This allows the conclusion that although being of the same chemical class these two drugs have a different mechanism of action.

When these compounds were assayed on a cell macrophage line, pentamidine significantly altered GR activity whereas the 4-methoxylated derivative did not. These results would suggest that the known toxicity of pentamidine could be accounted for by this inhibitory process.

Also, it seems that these mechanisms of action are involved in diminution of the percentage of macrophage infection observed "in vitro", corroborated by the fact that the methoxy-derivative is not toxic and does not interferes with cell host morphology.[36] Additionally, it was demonstrated that amidine derivatives also interfere with another defense system that of *L. amazonensis* promastigotes and axenic amastigotes, represented by the nitric oxide (NO) pathway.[37]

The results presented here are promising and the fact that TR is required for the parasites intracellular survival should constitute an interesting target for the study of new drugs, such as glutathione tripeptide analogs.

Acknowledgements

This work was supported by CAPES/FIOCRUZ-Brazil

References

[1] Channon, J.Y. and Blackwell, J. (1985) *Parasitology* **91**, 207–217.
[2] Bodgan, C., Rollinghoff, M. and Solbach, W. (1990) *Parasitol. Today* **6**, 183–187.
[3] Fairlamb, A.H. and Cerami, A. (1985) *Mol. Biochem. Parasitol.* **14**, 187–198.
[4] Fairlamb, A.H. and Cerami, A. (1992) *Ann. Rev. Microbiol.* **46**, 695–729.
[5] Shames, S.L., Fairlamb, A.H., Cerami, A. and Walsh, C.T. (1986) *Biochemistry* **25**, 3519–3526.
[6] Henderson, G.B., Fairlamb, A.H. and Cerami, A. (1987) *Mol. Biochem. Parasitol.* **24**, 39–45.
[7] Krauth-Siegel, R.L., Enders, B., Henderson, G.B., Fairlamb, A.H. and Schirmer, R.H. (1987) *Eur. J. Biochem.* **164**, 123–128.
[8] Jockers-Sherubl, M.C., Schimer, R.H. and Krauth-Siegel, R.L. (1989) *Eur. J. Biochem.* **180**, 267–272.
[9] Carnieri, E.G., Moreno, S.N.J. and Docampo, R. (1993) *Mol. Biochem. Parasitol.* **61**, 79–86.
[10] Fairlamb, A.H., Blackburn, P., Ulrich, P., Chait, B.T. and Cerami, A. (1985) *Science* **227**, 1485–1487.
[11] Taylor, M.D., Kelly, J.M., Chapman, C.J., Fairlamb, A.H. and Miles, M.A. (1994) *Mol. Biochem. Parasitol.* **64**, 293–301.
[12] Mukhopadhyay, R., Dey, S., Xu, N., Gage, D., Lightbody, J., Ouellete, M. and Rose, B.P. (1996) *Proc. Natl Acad. Sci. USA* **93**, 10383–10387.
[13] Krauth-Siegel, R.L. and Coombs, G.H. (1999) *Parasitol. Today* **15**, 404–409.
[14] D'Silva, C. and Daunes, S. (2002) *Expert. Opin. Investig. Drugs* **11**(2), 217–231.
[15] Girault, S., Davioud-Charvet, E., Maes, L., Dubremetz, J.F., Debreu, M.A., Landry, V. and Sergheraert (2001) *Bioorg. Med. Hem.* **9**, 837–846.
[16] Krauth-Siegel, R.L., Meiering, S.K. and Schmidt, H. (2003) *Biol. Chem.* **384**(4), 539–549.
[17] Ariyanayagam, M.R., Oza, S.L., Mehlert, A. and Fairlamb, A.H. (2003) *J. Biol. Chem.* **278**(30), 27612–27619.
[18] Cysne-Finkelstein, L., Aguiar-Alves, F., Temporal, R.M. and Leon, L.L. (1998) *Exp. Parasitol.* **89**, 58–62.
[19] Moreno, S.N.J., Carnieri, E.G.S. and Docampo, R. (1994) *Mol. Biochem. Parasitol.* **67**, 313–320.
[20] Toomik, R., Ekman, P. and Engström, L. (1992) *Anal. Biochem.* **204**, 311–314.
[21] Johnstone, A. and Thorpe, R. (1982) *Immunochemistry in Practice* (Blackwell Scientific Publications, Oxford), pp 1–3.
[22] Echevarria, A., Santos, L.H., Miller, J. and Mahmood, N. (1996) *Bioorg. Med. Chem. Lett.* **6**, 1901–1904.
[23] Krauth-Siegel, R.L., Jacoby, E.M. and Schirmer, R.H. (1995) *Meth. Enzymol.* **251**, 287–294.
[24] Canto-Cavalheiro, M.M., Echevarria, A., Araújo, C.A., Bravo, M.F., Santos, L.H., Jansen, A.M. and Leon, L.L. (1997) *Microbios* **90**, 51–60.
[25] Canto-Cavalheiro, M.M., Echevarria, A.A., Souza, M.A.S., Cysne-Finkelstein, L., Torres, M.A.A. and Leon, L.L. (2000) *Arzneim-Forsch/Drug Res.* **50**, 925–928.
[26] Hunter, W.N., Bailey, S., Habash, J., Harrop, S.J., Helliwell, J.R., Aboagye-Kwarteng, T., Smith, K. and Fairlamb, A.H. (1992) *J. Mol. Biol.* **227**, 322–333.
[27] Dumas, C., Ouellete, M., Tovar, J., Cunningham, M.L., Fairlamb, A.H., Tamar, S., Olivier, M. and Papadopoulou, B. (1997) *EMBO J.* **16**, 2590–2598.
[28] Krieger, S., Schwarz, W., Ariyanayagam, M.F., Fairlamb, A.H., Krauth-Siegel, R. and Clayton, C. (2000) *Mol. Microbiol.* **35**, 542–552.
[29] Tovar, J., Cunningham, M.L., Smith, A.C., Croft, S.L. and Fairlamb, A.H. (1998) *Proc. Natl Acad. Sci. USA* **95**, 5311–5316.
[30] Smith, K., Opperdoes, F.R. and Fairlamb, A.H. (1991) *Mol. Biochem. Parasitol.* **48**, 109–112.
[31] Augustyns, K., Amssoms, K., Yamani, A., Rajan, P.K. and Haemers, A. (2001) *Curr. Pharm. Des.* **7**, 1117–1141.
[32] Gomes-Cardoso, L., Echevarria, A., Aguiar-Alves, F., Cysne-Finkelstein, L., Canto-Carvalheiro, M.M., Genestra, M.S. and Leon, L.L. (1998) *Mem. Inst. Oswaldo Cruz* **I**, 312.

[33] Genestra, M., Echevarria, A., Cysne-Finkelstein, L. and Leon, L.L. (2001) *Arzneim-Forsch/Drug. Res.* **11**, 46–54.
[34] Zunino, F., Animati, F. and Capranico, G. (1995) *Curr. Pharm. Des.* **1**, 83–94.
[35] Temporal, R.M., PhD ThesisOswaldo Cruz Institute/FIOCRUZ-Brazil).
[36] Temporal, R.M., Cysne-Finkelstein, L., Echevarria, A., Souza, M.A.S., Sertã., M., da Silva-Goncalves, A.J., Pirmez, C. and Leon, L.L. (2002) *Arzneim-Forsch/Drug. Res.* **52**, 489–493.
[37] Genestra, M., Echevarria, A., Cysne-Finkelstein, L., Vignólio-Alves, L. and Leon, L.L. (2003) *Nitric Oxide: Biology and Chemistry* **8**, 1–6.