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Leishmania amazonensis trypanothione reductase: Evaluation of the effect of glutathione analogs on parasite growth, infectivity and enzyme activity

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Abstract

Trypanothione reductase (TR) is a major enzyme in trypanosomatids. Its substrate, trypanothione is a molecule containing a tripeptide (L-glutamic acid-cysteine-glycine) coupled to a polyamine, spermidine. This redox system (TR/Trypanothione) is vital for parasite survival within the host cell and has been described as a good target for chemotherapy anti-*Leishmania.* The use of tripeptides analogs of glutathione would result in a decrease in trypanothione synthesis and as a consequence in TR activity. In this work, besides the enzyme potential inhibition, it also evaluated the influence of those analogs on parasite growth and on its infective capacity. The results showed a significant effect on parasite growth and infectivity and in addition TR activity was highly inhibited. These results are very promising, suggesting a potential use of those analogs as therapeutic drugs against experimental diseases caused by trypanosomatids.

Keywords: *Trypanothione, trypanothione reductase,* Leishmania amazonensis*, glutathione analogs*

Introduction

Trypanothione reductase (TR) is a NADPH-dependent flavoenzyme found in parasitic protozoa, such as, *Trypanosoma* and *Leishmania* [1]. In most organisms, glutathione is an important antioxidant and levels of reduced glutathione are maintained by glutathione redutase (GR). However, trypanosomatids do not contain GR, instead they have a unique enzyme, trypanothione reductase (TR) (EC 1.6.4.8). TR catalyses the reduction of the disulphide of a glutathione-spermidine conjugate named trypanothione [N1- N8-bis (glutathionyl) spermidine]. The TR mechanism of action is identical to that of GR. The two enzymes have structural similarities; however, TR and GR have different substrate specificities [1–3]. Based on these data, it seems reasonable to design inhibitors of TR, which will not be able to inhibit GR. There are some relates in the literature concerning the use of glutathione analogs as potential inhibitors of TR [4–6]. Trypanothione reductase is crucial to the antioxidant defense of trypanosomatids and has been associated to the parasite infectivity by maintaining the infection within the macrophage [7,8]. In a previous work we described the activity of TR in *Leishmania amazonensis* promastigotes and axenic/lesion amastigotes and its association was corroborated to the parasite infectivity [9]. In the present work, using *L. amazonensis* promastigotes, several tripeptides (glutathione analogs) were tested in order to define its effects on parasite growth and enzyme activity. A screening with several tripeptides was carried out and the most effective compounds were choosing to evaluate those effects. The assayed compounds were able to decrease the parasite growth at least by 50%. Concerning TR inhibition, a significant decrease in the enzyme activity was observed. The parasite infectivity in the presence of those analogs was also investigated using mice

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peritoneal macrophages with a diminishing rate of infection (parasite x macrophage).

Materials and methods

Parasite

Leishmania amazonensis (MHOM/BR/77/LTB0016 strain) infective promastigotes were used and its virulence was maintained by subcutaneous injections in Balb/c mice every 2 months. Cultured promastigotes containing about 70% metacyclic forms are referred to as infective promastigotes [10]. In order to have promastigotes, amastigotes 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 (Schneider's Insect Medium, Sigma Cell Culture, USA), supplemented with inactivated fetal calf serum (10%) (FCS), 1 mmol/L, L-glutamine, 100 UI penicillin G and 100 μg/mL of streptomycin (Sigma) and incubated at 26°C.

Synthesis of the glutathione analogs

The tripeptides N-benzyloxycarbonyl-L-glutamyl(-benzyl)-L-Leu-Gly (ASC-I-74A), N-benzyloxycarbonyl-L-glutamyl(-benzyl)-L-Leu-L-Ala (ASC-I-74B), N-benzyloxycarbonyl-L-glutamyl(benzyl)-L-Val-Gly (ASC-I-75A) and N- benzyloxycarbonyl-L-glutamyl(benzyl)-L-Val-L-Ala (ASC-I-75B) were synthesized in four steps as described in Scheme 1 [5].

In a first step N-benzyloxycarbonyl-L-glutamic acid -benzyl ester 2 was esterified with N-hydroxysuccinimide using N, N′-dicyclohexylcarbodiimide (DCC). Using standard protocols, the ester 2 was coupled to either L-Leucine or L-Valine, affording dipeptides 3 or 3′, respectively. Finally, esterification of each dipeptide as previously described, afforded the esters 4 and 4′, which were subsequently coupled to Glycine or L-Alanine, providing the four tripeptides ASC-I-74A, ASC-I-74B, ASC-I-75A and ASC-I-75B in good overall yields.

Cellular fractionation

Soluble fractions (SF) of infective promastigotes (up to 5 passages in culture) were used for the enzyme assay, as follows: parasites 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 (Sigma Chemical Co. USA), lysed in a Dounce homogenizer, centrifuged at 12,500*g*/15 min and the supernatant considered the soluble fraction (SF) [11]. Protein (mg/mL) concentrations were measured through spectrophotometry according to Johnstone and Thorpe (1992) [12].

Glutathione analogs and parasite growth

Glutathione analogs (ASC-I-74A, ASC-I-74B, ASC-I-75A and ASC-I-75B) were dissolved in DMSO (the highest concentration was 1.4%, which was not hazardous to the parasites) and added to parasites suspensions in a concentration range of 320 μg/mL to 10 μg/mL. After 24 h of incubation, the parasites were counted and compared to the controls, containing DMSO and parasites alone. The drug concentration corresponding to 50% of parasite growth inhibition was expressed as the LD_{50}. In parallel another screening with the same glutathione analogs but without both benzyl groups (ASC-I-9, ASC-I-99, ASC-II-9V and ASC-I-98V) was realized, but all the compounds did not show any effect on parasite growth and were not used to assay TR activity.

TR activity assay

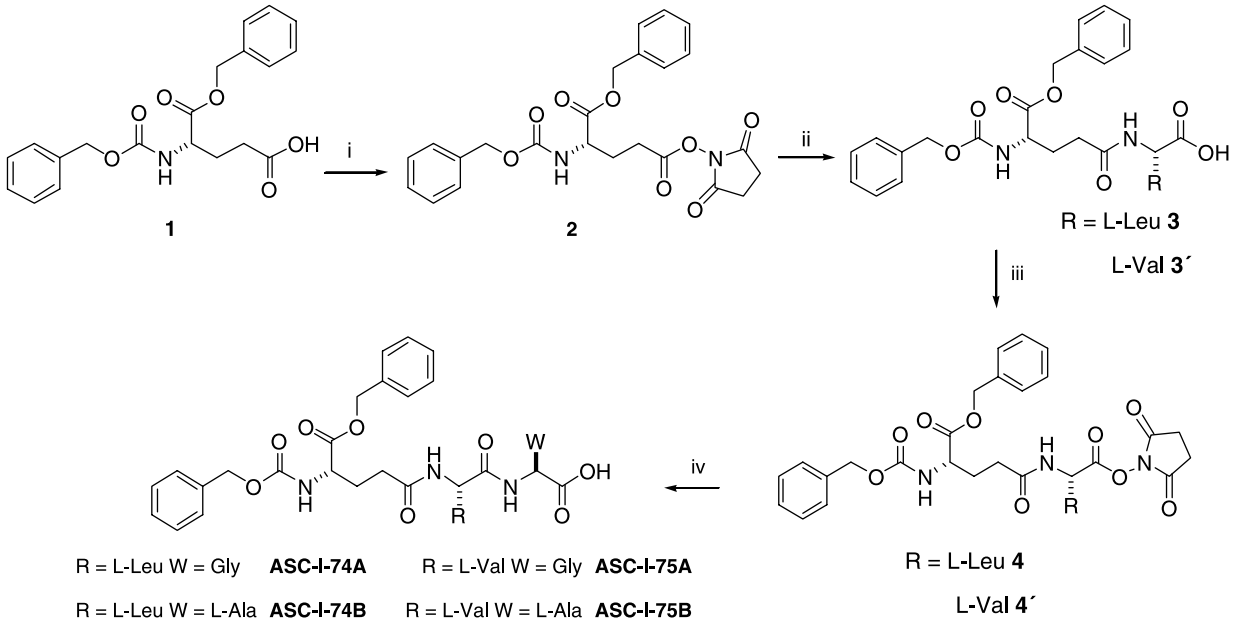
To evaluate the effect of analogs on TR activity in *L. amazonensis*, the infective promastigotes soluble fraction was used. TR activity was detected spectrophotometrically by measuring NADPH consumption at 340 nm [12]. The assay mixture contained 40 mmol/L HEPES, pH 7.5, 1 mmol/L EDTA, 100 μmol/L NADPH, parasite samples (1 mg/mL of protein) and the analogs were added to the reaction mixture in concentrations equivalent to their LD_{50} values over a 24 h period. Controls were used without the analogs. To be sure that NADPH consumption was associated to TR activity, an assay was carried out in which it was added an excess (500 μM and 1000 μM) of oxidized glutathione (GSSG), as described before [9,13]. The reduced trypanothione T $(SH)_2$ formed will react with the excess of GSSG, producing more oxidized trypanothione $T(S)_2$ and increasing the NADPH consumption, according to the scheme:

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

$$T(SH)_2 + GSSG(\text{excess}) \rightarrow T(S)_2 + GSH(\text{non-enzymatic reaction}).$$

Evaluation of the effect of glutathione analogs on parasite infectivity

In attempt to verify the effect of glutathione analogs on parasite infectivity, an *in vitro* assay was realized, as follows: *L. amazonensis* promastigotes were cultured in Schneider's medium containing 10% fetal calf serum



Scheme 1. Synthesis of glutathione analogs. Synthesis of tripeptides ASC-I-74A, ASC-I-74B, ASC-I-75A, ASC-I-74B: (i) N-α-benzyloxycarbonyl-L-glutamic acid α-benzyl 1, N-hidroxysuccinimide, dry dioxane, DCC, 0°C, 12 h, 98%; (ii) R = L-Leu or L-Val, H_2O, Et_3N, ester 2, THF, rt, 12 h, 76%; (iii) N-α-benzyloxycarbonyl-L-glutamyl(α-benzyl)-L-Leu 3 or L-Val 3′, N-hidroxysuccinimide, dry dioxane, DCC, 0°C, 12 h, 96%; (iv) W = Gly or L-Ala, H_2O, Et_3N, ester 4 or 4′, THF, rt, 12 h, ASC-I-74A: 85%; ASC-I-74B: 81%; ASC-I-75A: 83%; ASC-I-75B: 76%.

(FCS) (Cultilab), at 26°C, and after 3 days of growth, a parasite concentration of 2×10^6 parasites/mL were treated with glutathione analogs in concentrations corresponding to $LDs_{50}/24$. After 24 h of incubation, parasites were washed and added to cell culture slides (Lab Tek/8 wells), containing Balb/c mice peritoneal macrophages (5×10^6/mL) in RPMI medium, as described elsewhere [14]. After 24 and 48 h incubation at 37°C and under an atmosphere of CO2 (5%), the slides were rinsed in PBS pH 7.2, dried and fixed in methanol (Merck)/4 min., and submitted to Instant-prov dye and evaluated in an optical microscope (Zeiss, 100x). The percentage of infection and the number of parasites/macrophages were determined and compared to the controls of infection, without the analogs and the macrophage alone. All experiments were carried out in triplicate [14].

Statistical analysis

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

Results

Parasite growth inhibition

All compounds were evaluated *in vitro* on *L. amazonensis* promastigotes growth. The compounds containing two benzyl groups linked to glutamyl moiety were effective (ASC-I-74A, ASC-I-75A, ASC-I-74B and ASC-I-75B). On the other hand, the other series without benzyl groups (ASC-I-9, ASC-I-99, ASC-II-9V and ASC-I-98V) were not effective, with IC > 320 μg/mL and were not used in further experiments (Table I).

TR inhibition assay

The evaluation of TR activity was based on the NADPH consumption. A decrease in this consumption was observed indicating that TR activity was inhibited in the presence of 74A, 74B, 75A, 75B ($p < 0.05$) (Figure 1).

Effect of glutathione analogs on macrophage infection

Promastigotes treated with phenylated tripeptide analogs were used to infect mice peritoneal

Table I. Effect of glutathione analogs on *Leishmania amazonensis* infective promastigotes. Parasites were incubated with/without the analogs and after 24 h the LDs_{50} were calculated.

Compound	LD_{50} μg/mL	LD_{50} μM
ASC-I-74A	188,6 ± 5,0	348,6
ASC-I-74B	134,7 ± 4,8	242,7
ASC-I-75A	115,7 ± 3,8	219,5
ASC-I-75B	174,4 ± 2,9	322,3
ASC-I-9	>320	ND
ASC-I-99	>320	ND
ASC-II-9V	>320	ND
ASC-I-98V	>320	ND

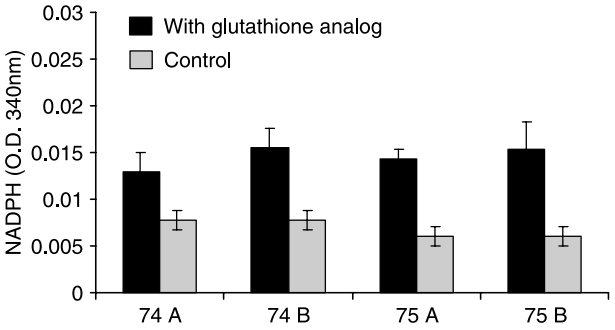


Figure 1. Effect of glutathione analogs on the activity of TR of *Leishmania amazonensis* infective promastigotes. The enzyme activity was measured by NADPH consumption.

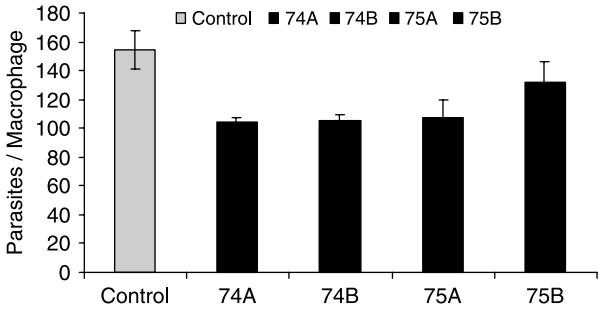


Figure 3. Effect of glutathione analogs on parasite x macrophage interaction, after 48 h of incubation.

macrophage. Parasites were able to enter the macrophage and maintain the infection. It is important to notice that after 24 h incubation, while parasites treated with compounds 74A, 75A and 74B infected macrophages in a similar way of the controls, in the absence of analogs ($p > 0.05$), parasites treated with compound 75B showed a significant increase in the growth inside the macrophage ($p < 0.05$) (Figure 2). On the other hand, after 48 h, all groups presented a decrease in the infection rate, comparing to the control ($p < 0.05$) (Figure 3). Also, concerning parasites in the presence of compound 75B, in comparison to the 24 h results, a similar behavior was observed ($p > 0.05$), with no increase in the infection (number of parasites/macrophages).

Discussion

Trypanothione is a molecule containing glutathione (L-glutamic acid -L-cysteine-L-glycine), linked to the terminal amino group of the polyamine spermidine. It is already known that glutathionyl spermidine synthetase (GSS) and trypanothione synthetase (TS) are enzymes involved in trypanothione synthesis. An inhibition of one of those enzymes will result in a lack of trypanothione and in a consequence there will be no TR activity in the absence of substrate. However, there are few data in literature about the inhibition of trypanothione biosynthesis [15–18]. Studies related to GSS and TS inhibitors, using glutathione analogs, showed that the substitution of the L-Cys in glutathione by hydrophobic amino acids, as L-leu and L-val, result in efficient non-competitive inhibitors of these enzymes. However, overall, these inhibitors did not appear to be efficient against *Leishmania* sp., probably due to the action of proteases and to the difficulty of the compounds to penetrate through cellular membrane [19,20]. Taking these inhibitors as prototypes, two series of tripeptids were synthesized; in one series, beside the change of aminoacids, benzyl esther radicals were included on C and N terminal groups of the glutamic acid (ASC-I-74A, ASC-I-75A, ASC-I-74B and ASC-I-75B), which increase the lipophilicity of the molecule. These series presented a very significant effect against *L. amazonensis* TR, measured by a lesser NADPH consumption by the enzyme. On the other hand, the second series which was similar to the first one, but without protective benzyl esther groups (ASC-I-9, ASC-I-99, ASC-II-9V, ASC-I-98V) presented no activity against the parasite. These data suggest that these protective radicals contributed to an increase of the drug biodisponibility, associated to a higher lipophilicity of the molecule, since the same tripeptids with the free functional groups (without protection) were not active.

One physiological function of the trypanothione/TR system is the detoxification of reactive oxygen species produced by the host cell and further parasite survival. TR is able to accept and process a variety of glutamyl replacement substrates, however, omission of the glutamyl-NH^+ groups destroyed enzyme activity [21]. In this work the modifications were done in the glutamyl moiety and TR activities was only decreased, corroborating these data. Furthermore, here the modifications were done before the association of glutathione and spermidine. We are not able to define whether a modified glutathione has the capacity to join spermidine to form trypanothione within the parasite,

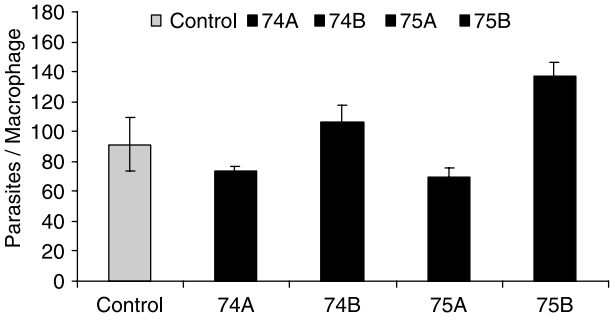


Figure 2. Effect of glutathione analogs on parasite x macrophage interaction, after 24 h of incubation.

or what it means if the glutathionyl spermidine synthetase is able to recognize the modified substrate, which would result in a diminishing of TR actitivity, as a consequence of a decrease of trypanothione.

The analogs induced a decrease in TR activity and promastigotes growth, but macrophage infection was efficient suggesting that parasites used another metabolic pathway in order to survive well within the macrophages, or the remaining enzyme activity was enough to maintain the infection.

The use of glutathione analogs represents a potential inhibition mechanism of the growth, and TR activity of the parasite reinforced that tripanothione/TR system could be a target for chemotherapy of leishmaniasis.

Acknowledgements

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