Bioorganic & Medicinal Chemistry 20 (2012) 1760–1766

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Investigation of trypanothione reductase inhibitory activity by 1,3,4-thiadiazolium-2-aminide derivatives and molecular docking studies

Raquel F. Rodrigues [a,*], Denise Castro-Pinto [a], Aurea Echevarria [b], Camilla M. dos Reis [b], Catarina N. Del Cistia [b], Carlos Mauricio R. Sant'Anna [b], Filipa Teixeira [c], Helena Castro [c], Marilene Canto-Cavalheiro [a], Leonor L. Leon [a], Ana Tomás [c]

[a] *Fundação Oswaldo Cruz, Instituto Oswaldo Cruz, Laboratório de Bioquímica de Tripanosomatídeos, Av. Brasil, 4365, Pavilhão 26, sala 405, Manguinhos, Rio de Janeiro, RJ 21045-900, Brazil*
[b] *Departamento de Química, Universidade Federal Rural do Rio de Janeiro, Seropédica, Brazil*
[c] *Instituto de Biologia Molecular e Celular, Universidade do Porto, Porto, Portugal*

ARTICLE INFO

Article history:
Received 27 September 2011
Revised 28 December 2011
Accepted 5 January 2012
Available online 14 January 2012

Keywords:
Trypanothione reductase
Mesoionic compounds
Leishmania sp.
Trypanosoma cruzi

ABSTRACT

The biological activities of a series of mesoionic 1,3,4-thiadiazolium-2-aminide derivatives have been studied. The most active compounds (MI-HH; MI-3-OCH_3; MI-4-OCH_3 and MI-4-NO_2) were evaluated to determine their effect on trypanothione reductase (TryR) activity in *Leishmania* sp. and *Trypanosoma cruzi*. Among the assayed compounds, only MI-4-NO_2 showed enzyme inhibition effect on extracts from different cultures of parasites, which was confirmed using the recombinant enzyme from *T. cruzi* (*Tc*TryR) and *Leishmania infantum* (*Li*TryR). The enzyme kinetics determined with *Li*TryR demonstrated a non-competitive inhibition profile of MI-4-NO_2. A molecular docking study showed that the mesoionic compounds could effectively dock into the substrate binding site together with the substrate molecule. The mesoionic compounds were also effective ligands of the NADPH and FAD binding sites and the NADPH binding site was predicted as the best of all three binding sites. Based on the theoretical results, an explanation at the molecular level is proposed for the MI-4-NO_2 enzyme inhibition effect. Given TryR as a molecular target, it is important to continue the study of mesoionic compounds as part of a drug discovery campaign against Leishmaniasis or Chagas' disease.

1. Introduction

Parasitic protozoa of the family Trypanosomatidae are the causative agents of many significant tropical diseases, including African trypanosomiasis, Chagas' disease, and Leishmaniasis. *Trypanosoma cruzi* is a protozoan parasite from the order Kinetoplastida that causes Chagas' disease. As a result of sustained campaigns of vector control, the number of new infections in endemic areas has decreased, but the disease still affects 16–18 million people in Latin America and belongs to the group of neglected diseases as defined by the World Health Organization (WHO).[1] Only two drugs, nifurtimox and benznidazol, are currently available to treat this disease, and these remain unsatisfactory due to their toxic side effects and limited efficacy in the chronic phase of the infection.[2,3] Leishmaniasis is caused by numerous parasitic protozoan subspecies of the genus *Leishmania* and is endemic in 88 countries on four continents. In Brazil, the number of cases has been increasing, both in magnitude and geographic expansion.[4] Leishmaniasis covers a variety of forms, from cutaneous ulcers, which causes self-healing lesions, to visceral leishmaniasis, which is fatal if left untreated. Among the different therapeutic alternatives available for leishmaniasis, the WHO recommends the pentavalent antimonies as first-choice medicines, primarily meglumine antimoniate.[5,6] An increase in the incidence of drug resistance has been reported, requiring the use of prohibitively expensive drugs, such as liposomal amphotericin B.[5] The currently available chemotherapeutic agents against these diseases are still inadequate. Hence, there is an urgent need for the development of new, cost-effective drugs with minimal side effects. Therefore, the identification of new targets for anti-trypanosomatids is incredibly important.[7]

The enzyme trypanothione reductase (TryR) was described in 1985[8] and is a validated drug target in trypanosomatids, as it was shown to be essential for the survival of these parasites by protecting them against oxidative stress.[8,9] This enzyme is dependent on NADPH and catalyzes the reduction of trypanothione disulfide [$T(S)_2$] dithiol to trypanothione [$T(S)_2$], triggering a cascade of events responsible for the neutralization of reactive oxygen species.[9] In addition to playing a pivotal role in the parasite, another characteristic that makes TryR a potential target for

* Corresponding author. Tel.: +55 21 3865 8225.
E-mail address: rakelbio@gmail.com (R.F. Rodrigues).

0968-0896/$ - see front matter © 2012 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2012.01.009

antiparasitic drugs is its significant structural difference from glutathione reductase (GR), the enzyme with the corresponding function in humans.[10,11]

The chemistry of mesoionic rings, especially their use as masked dipoles, has been a fruitful area of research since the late 50s.[12] Mesoionic compounds are a special class of heterocycles with special features that contribute to several biological activities. They possess a betaine-like character with a partial positive charge on the heterocyclic ring, which generally includes five members and is balanced by a negative charge located on an exocyclic atom.[12,13] The large separation between the charged regions leads lo large dipole moments of about 4–5 D.[12,14] These properties suggest the possibility of interactions with biomolecules such as proteins and DNA, and the overall neutral character of these compounds enables them to cross biological membranes.

Our previous studies have proven that mesoionic derivatives of the 1,3,4-thiadiazolium-2-aminide class inhibit the in vitro growth of *Leishmania amazonensis*, *Leishmania braziliensis*, *Leishmania chagasi*[15,16] and *Trypanosoma cruzi*.[17] The activity of two mesoionic derivatives on the *L. amazonensis* infection in BALB/c mice, including the decrease of lesion size and parasitic load, has also been reported.[18] However, other biological activities have been revealed, including anti-inflammatory, analgesic, antibacterial, antifungal, and antitumor activities.[18–21] To continue our study of mesoionic compounds, especially our investigation into the mechanism of action, we sought to elucidate the target of mesoionic derivatives on *Leishmania* sp. and *Trypanosoma cruzi*. Three species of *Leishmania* were selected to this work, two species native to the New World, *Leishmania (L) amazonensis* and *Leishmania (V) braziliensis*, and another native to the Old World, *Leishmania (L) infantum*. The effects of mesoionic derivatives (Fig. 1) on TryR from parasite extracts and on recombinant enzymes from *L. infantum* and *T. cruzi* were evaluated.

2. Materials and methods

2.1. Parasites

Parasites of *L. infantum* (MHOM/MA67ITMAP263 strain) were used in all experiments. *L. amazonensis* (MHOM/BR/LTB0016 strain), *L. braziliensis* (MCAN/BR/98/R619) and *T. cruzi* (Y strain) were used to obtain soluble parasite extracts to analyze the effects of mesoionic derivatives on TryR.

2.2. *Leishmania* sp. cultivation

L. infantum promastigotes were cultivated in RPMI medium (GlutaMAX, Invitrogen) at 26 °C and supplemented with 10% fetal calf serum (FCS). *L. amazonensis* promastigotes were cultivated in Schneider's *Drosophila* medium, pH 7.2, supplemented with 2 mm L-glutamine, 50 mm HEPES, 35 U/mL penicillin, 35 μg/L streptomycin, 2% human urine, and 10% FCS, at 27 °C in a Biochemistry Oxygen Demand (BOD) incubator. *L. braziliensis* promastigotes were cultivated at the same conditions but with 20% FCS.

2.3. *Trypanosoma cruzi* cultivation

Epimastigote forms of the Y strain of *T. cruzi* were maintained in liver infusion tryptose (LIT) medium, pH 7.2, supplemented with 10% FCS and maintained at 27 °C in a BOD incubator.[22,23] The epimastigotes were passaged weekly.

2.4. Soluble extract preparation of *Leishmania* sp. and *T. cruzi*

Soluble extracts were obtained from infective promastigotes and epimastigotes cultures. Parasites were removed from their respective medium by centrifugation at 500*g*/10 min. Pellets were resuspended in PBS, pH 7.2; they were then centrifuged two more times under the same conditions and further added to the final buffer, which contained 40 mM HEPES and 1 mM EDTA. The material was lysed in a Dounce-type homogenizer and centrifuged at 12,500*g*/15 min. The supernatant was considered to be the soluble extract that contained trypanothione reductase (TryR).[24] The whole preparation of soluble extract was carried out at 8–12 °C of temperature to avoid damage to the enzyme. The protein concentration of the soluble extract was assessed using a NanoDrop 1000 spectrophotometer (Thermo Scientific). All samples were aliquoted and stored at −70 °C until analysis.

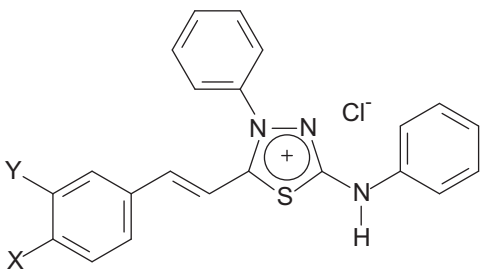
2.5. Mesoionic derivative synthesis

2.5.1. General procedure for the preparation of 4-phenyl-5-(4′-X- or 3′-Y-styryl)-1,3,4-thiadiazolium-2-phenylamine chlorides

The compounds, 4-phenyl-5-(4-H-, 4-OCH_3-, 3-OCH_3-, or 4-NO_2-styryl)-1,3,4-thiadiazolium-2-phenylamine chlorides, were fully characterized previously,[15] by infrared (IR), ^1H, ^{13}C NMR spectroscopy and mass spectrometry. The mesoionic derivatives were synthesized by the coupling of the corresponding freshly prepared 4′- or 3′-substituted *styryl* chlorides (10 mmol) which were added to a stirred solution of 1,4-diphenylthiosemicarbazide (10 mmol) in dry 1,4-dioxane (20 mL) at room temperature. After standing 24–48 h, the products were separated by vacuum filtration, washed with dry 1,4-dioxane and recrystallized from ethanol/dichloromethane (1:1 v/v) to yield yellow crystals.

2.6. Enzymatic assays in the soluble extract of *Leishmania* sp.

The capacity of mesoionic derivatives to inhibit TryR activity was evaluated using the equivalent of 1 mg/mL of soluble protein extract. Four mesoionic derivatives (MI-HH; MI-3-OCH_3; MI-4-OCH_3 and MI-4-NO_2) were pre-incubated with the soluble extracts (derivatives at 1 μM) for 10 min, then 100 μM NADPH, 40 mM HEPES and 1 mM EDTA, pH 7.5, was added. The assay was initiated in a spectrophotometer (Shimadzu Corporation, Japan) at 340 nm to



X = H; Y = H (**MI-HH**)
X = OCH_3; Y = H (**MI-4-OCH_3**)
X = H; Y = OCH_3 (**MI-3-OCH_3**)
X = NO_2; Y = H (**MI-4-NO_2**)

Figure 1. Chemical structures of mesoionic derivatives of 1,3,4 thiadiazolium-2-aminide class.

check the total value of NADPH during the 1 min of reading, without alterations; 100 mM trypanothione disulfide ($T(S)_2$, Bachem) was then added to optimize and direct the reaction to NADPH consumption by TryR. All reactions were performed at 25 °C, in a total volume of 300 μL. After that, readings were started quickly for 100 s more. The inhibition percentage was calculated based on the decay of optical density, which reflects NADPH oxidation; hence, optical density represents NADPH consumption by TryR. This decay was given for optical density differences (ΔOD) after the first 5 s after the addition of $T(S)_2$.[24–27]

2.7. Enzymatic and kinetics assays with recombinant TryR

The assay was performed with *L. infantum* recombinant trypanothine reductase (*Li*TryR) and *T. cruzi* recombinant trypanothine reductase (*Tc*TryR). An assay mixture consisted of *Li*TryR that had been incubated with three different concentrations (0.5, 1 and 2 μM) of two mesoionic derivatives (MI-HH; 4-NO_2) for 10 min. Briefly, the reaction mixtures, 40 mM HEPES, 1 mM EDTA, pH 7.5, containing 100 μM NADPH, were prepared in a total volume of 300 μL. Those mixtures were monitored at 340 nm for 1 min until a constant baseline was obtained, and the reaction was then started by adding five different concentrations of $T(S)_2$ (25, 50, 100, 200 and 500 μM). This decay was given for optical density differences (ΔOD), which correlated with NADPH consumption. All reactions were performed at 25 °C and monitored with a Shimadzu UV-2401 PC spectrophotometer (Shimadzu Corporation). Each data set was fitted by nonlinear regression to the Michaelis–Menten equation. The resulting individual fits were examined as Lineweaver–Burk transformations, and the graphs inspected for diagnostic inhibition patterns. The entire data set was then globally fitted to the appropriate equation (competitive, mixed or uncompetitive inhibition). The same rationale was followed for *Tc*TryR, but only one concentration each of the mesoionic compounds (1 μM) and $T(S)_2$ (50 μM) were used, and no enzyme kinetic was determined.

2.8. Molecular modeling

Molecular docking studies were implemented in order to compare the interaction of the mesoionic compounds at the molecular level with the TryR enzyme of the four parasite species: *Leishmania amazonensis*, *L. braziliensis*, *L. infantum* and *Trypanosoma cruzi*.

2.8.1. *Leishmania infantum* TryR (*Li*TryR)

For the docking studies into the *L. infantum* TryR, it was used the crystal structure obtained from Protein Data Bank site (PDB[28]), with the accession code 2W0H.[29] The resolution was relatively low (3.00 Å), but this structure was chosen for having the cofactors flavin adenine dinucleotide (FAD) and nicotinamide adenine dinucleotide (NADPH, in the reduced form) complexed in their respective binding sites, which are putative binding sites for the mesoionic compounds. The binding sites are set in a more open conformation by the presence of the cofactors, which is expected to facilitate the docking procedure. The cofactors, water and sulfate molecules were deleted from the 2W0H structure before the mesoionic compounds docking was performed.

2.8.2. *Trypanosoma cruzi* TryR (*Tc*TryR)

The docking study into the *T. cruzi* TryR was done with the X-ray structure deposited in PDB with accession code 1BZL.[11] Water molecules were also deleted before the docking procedure.

2.8.3. *Leishmania braziliensis* TryR (LbTryR)

It was necessary the construction of a 3D model of a *L. braziliensis* TryR, because there is no crystal structure available for this protein. The primary sequence of *L. braziliensis* TryR was obtained in the Swiss Prot/TrEMBL (http://www.expasy.ch/sprot/[31,32]) server with the access code A4H480-1 (UniParc). The template for the modeling procedure was the crystal structure of the *L. infantum* TryR complexed with FAD (PDB accession code 2JK6[29]), which was selected with the Swiss-Model BLAST tool[30] from the Swiss-Model protein-modeling server (http://www.expasy.ch/swissmod/SWISS-MOD-EL.html). The template protein has a 2.95 Å resolution and its sequence identity with *L. braziliensis* TryR is 83,7%. Because of this high sequence identity, we adopted a direct residues replacement procedure with the Deep View 3.7 program[33,34] on the X-ray structure as the strategy for construction of the 3D model. The sequences of both enzymes were aligned and *L. braziliensis* TryR diverging residues with the *L. infantum* TryR sequence were then replaced with the 'mutate' tool. The initial rotamer of the mutated residues were selected in order to improve the number or hydrogen bonds and reduce the number of atom clashes. The final structure was then submitted to energy minimization with GROMOS96[35] as implemented in the 'energy minimization' tool of the program.

2.8.4. *Leishmania amazonensis* TryR (*La*TryR)

There is no crystal structure available for this protein, as *L. braziliensis* TryR. We employed the model obtained previously by our group[25] using the *Crithidia fasciculata* TryR (PDB accession code 1FEC[48]) X-ray structure as a template.

Ligand structures were energy minimized with the PM3 semiempirical method[36] available in the Spartan'08 program (Wavefunction, Inc.) and saved in the SYBYL MOL2 file format.

The docking of the ligands into the TryR structures was implemented with the GOLD 5.0 program (CCDC Software Ltd), an efficient genetic algorithm for docking flexible ligands into protein binding sites. Hydrogen atoms were added to the proteins based on ionization and tautomeric states inferred by the program. The number of genetic operations (crossover, migration, mutation) in each run during the searching procedure was set to 100.000. The program optimizes hydrogen-bond geometries by rotating hydroxyl and amino groups of amino acid side chains. All scoring functions available in the program were used for calculations: GoldScore,[37] ChemScore,[38] ASP[39] and ChemPLP.[40,41]

The TryR contains a active binding site for $T(S)_2$ and two allosteric binding sites for NADPH and FAD binding, which were also explored in the docking procedure. The binding site was defined with a 20 Å radius from selected residues located at the center of each site. In the active binding site, the mesoionic compounds were docked containing $T(S)_2$ at the same time, because it was determined experimentally that the inhibition was non-competitive. The three binding sites residues were analyzed and compared between the *L. amazonensis*, *L. braziliensis*, *L. infantum* and *T. cruzi* enzymes, looking for explanations for activity differences between the compounds under study.

2.9. Statistic analysis

Significance was determined using a non-paired Student's *T*-test. Differences were considered to be significant when $p < 0.05$. Each experiment was performed in triplicate.

3. Results

3.1. Trypanothione reductase activity assay in soluble parasite extracts

The aim of the assay was to determine the effect of four mesoionic derivatives on TryR activity. Initially, this effect was evaluated in

soluble extracts from the late log phase of *Leishmania amazonensis* promastigotes (Fig. 2A). The reaction was followed by NADPH consumption in the presence/absence of the compounds, 5 s after $T(S)_2$ addition. The control (in the absence of mesoionic derivatives) was considered to be 100% TryR activity. The addition of mesoionic derivatives (MI-HH, MI-4-OCH_3 and MI-3-OCH_3) did not modify NADPH consumption in this TryR activity assay, except the derivative MI-4-NO_2 (1 μM), which was able to inhibit 76% (p <0.005) of NADPH consumption by *L. amazonensis* extracts compared with the control. Therefore, the same assay was carried out with other soluble extracts from the other parasites, using the late log phase of *L. infantum*, *L. braziliensis* promastigotes and *T. cruzi* epimastigotes (Fig. 2B). MI-4-NO_2 at 1 μM was able to inhibit NADPH consumption by TryR in parasites extracts, at 70% in *L. infantum*, 69.5% in *L. braziliensis* and 83% in *T. cruzi* (p <0.005). These results strongly indicate that TryR could be a target for MI-4-NO_2. The same assay was also performed with MI-HH, as benchmark.

3.2. Trypanothione reductase activity assay in recombinant enzymes from *L. infantum* and *T. cruzi*

From the results obtained from the TryR activity assay in soluble extracts of parasites, NADPH consumption assays were conducted using recombinant enzymes from *L. infantum* (*Li*TryR) and *T. cruzi* (*Tc*TryR). These assays were followed for 100 s, in which NADPH consumption was analyzed after the addition of 50 μM $T(S)_2$ to the reaction mixture. It is important to note that up to 60 s of evaluation, there was no consumption or any significant change in the NADPH amount before the addition of substrate (Fig. 3). After this addition, a fast NADPH consumption in the first 5 s of reaction was observed. In parallel, assays were conducted in the presence of DMSO, for which no significant changes were observed in NADPH consumption. Pre-incubation of 1 μM of MI-4-NO_2 with both *Li*TryR and *Tc*TryR significantly decreased the absorbance decay in the first 5 s, with inhibition of 76% of *Li*TryR and 69% of *Tc*TryR (p <0.005). The addition of 1 μM of MI-HH did not alter NADPH consumption in comparison with the control.

The enzyme kinetics of *Li*TryR were analyzed to confirm the mode of action of MI-4-NO_2 and MI-HH. Three concentrations were used for both compounds. The rates of such enzyme reactions could be analyzed by the Michaelis–Menten approach and algebraically transformed into forms that are more useful in practical studies. For that reason, these kinetic data are shown in the Figure 4 as a Lineweaver–Burk graph. In Figure 4A, the MI-4-NO_2 derivative was used as the *Li*TryR inhibitor on enzyme kinetics, effectively decreasing their activity. This effect on the enzyme was demonstrated by lower values of V_{max} (maximum reaction rate; graph vertical axis) and little or no apparent effect on K_m (Michaelis constant; graph horizontal axis), which are the characteristic effects of a noncompetitive inhibition. The IC_{50} (inhibition concentration 50%) for *Li*TryR was estimated in 1.63 ± 0.06 μM to MI-4-NO_2. However, as Figure 4B shows, there were no alterations when the three concentrations of MI-HH were tested in the same assay with *Li*TryR.

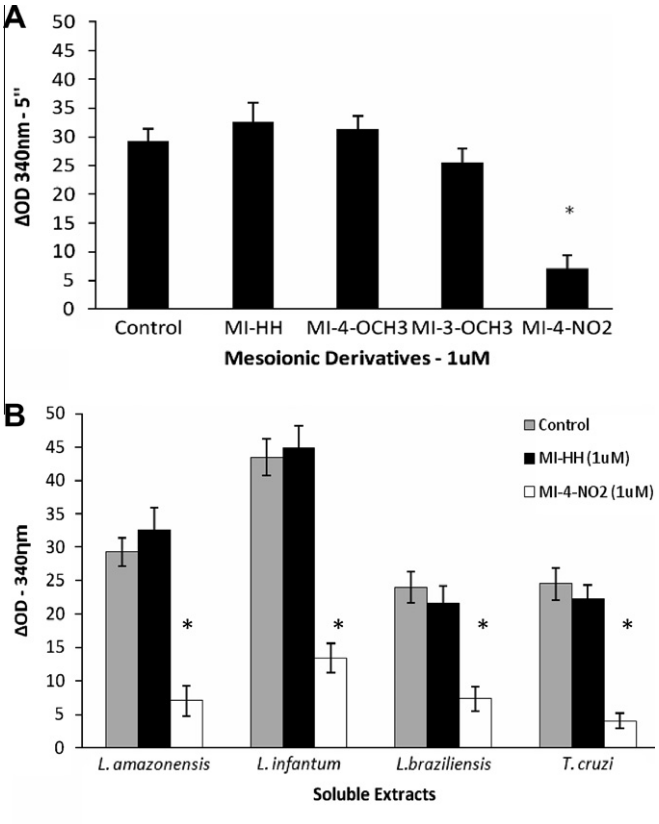


Figure 2. TryR inhibition in soluble extracts from parasites by mesoionic derivatives at 1 μM concentration. NADPH consumption by TryR activity was evaluated after 5″ of $T(S)_2$ addition; control means the maximum amount of NADPH consumption in soluble extract (*p <0.005). A. NADPH consumption by TryR in *L. amazonensis* extract in the presence of all compounds. B. NADPH consumption by TryR in different parasite extracts of *Leishmania* promastigotes and epimastigotes of *T. cruzi* in the presence of MI-HH and MI-4-NO_2.

3.3. Docking studies

In order to identify at the molecular level possible reasons for the different enzyme inhibition profiles of the mesoionic compounds, a docking study with the TryR of the four parasite species was implemented. The molecules were evaluated in the neutral form, because of the conditions used in the experimental procedure (neutral pH). As TryR is a FAD-dependent oxidoreductase, which utilizes NADPH as an electron donor, it contains binding sites for FAD and NADPH, besides the substrate binding site. All four docking functions were able to effectively dock these ligands into their respective binding sites. We present here the results obtained with one of these functions, ASP. ASP is an atom–atom potential derived from a database of protein–ligand complexes and it was demonstrated to predict the correct binding modes of a list of 139 druglike compounds with a success rate of 72%.[39]

The first site investigated with the docking method was the substrate binding site. Since the kinetic data indicated a non-competitive *Li*TryR inhibition profile by MI-4-NO_2, docking runs were also performed with the TSST binding site containing the substrate molecule. A number of poses for each ligand were obtained and scored and the best-ranked pose in each TryR was chosen for further analysis. The fitness functions of the GOLD program give fitness scores that are dimensionless. In each case, the scale of the score is a guide of how good the docking pose is; the higher the score, the better the docking result probably is. The docking results are summarized on Table 1.

Next, docking runs were implemented into the TryR FAD and NADPH allosteric binding sites. Because of the close proximity between both sites, it was necessary to previously dock the FAD molecule into its binding site before the docking runs of the ligands into the NADPH binding site were executed; similarly, the ligands were docked into the FAD binding site of the NADPH/TryR binary complex. In addition, as the substrate could possibly exert some influence in the docking of the mesoionic compounds into the FAD binding site due to its closeness to the TSST binding site, we also made docking runs with the enzyme containing both TSST and NADPH (Table 2).

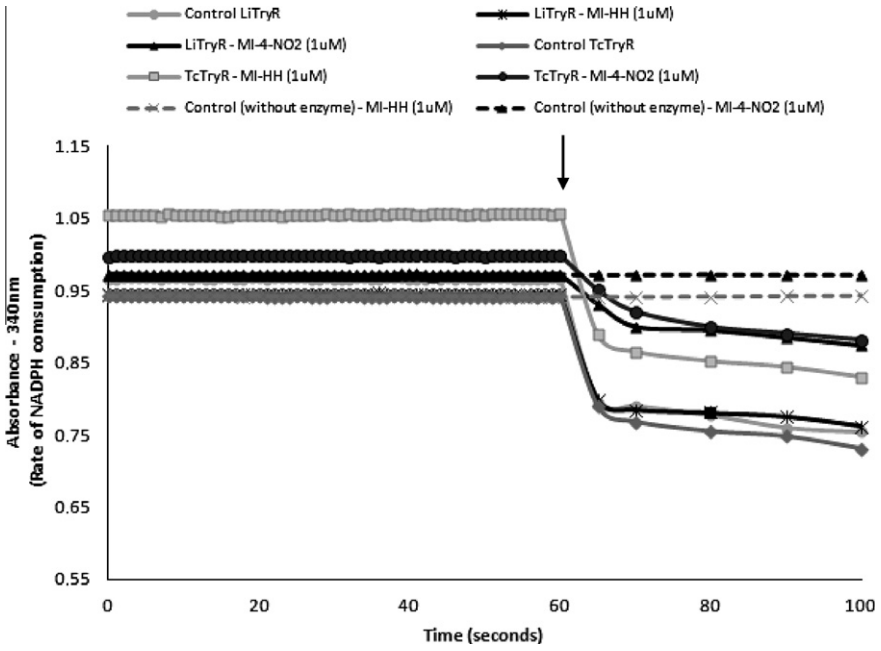


Figure 3. *Li*TryR and *Tc*TryR inhibition by MI-4-NO_2. Activity of TryR on $T(S)_2$ (50 μM), followed by monitoring NADPH consumption at 340 nm. The reaction mixture contained NADPH, TryRs and MI derivatives. The arrow indicates the moment when $T(S)_2$ was added after 60 s of monitoring. The mesoionic derivatives tested were MI-4-NO_2 (triangles and black circles) and MI-HH (asterisks and squares). Controls are gray circles for *Li*TryR and gray diamonds for *Tc*TryR. Controls without enzyme (*Li*TryR or *Tc*TryR) using MI compounds are dashed lines, MI-4-NO_2 (black triangles) and MI-HH (gray asterisks).

4. Discussion

Synthetics compounds or compounds from natural sources have been investigated as selective inhibitors of trypanothione reductase in several previous studies.[11,26,41,24,23,42] Trypanosomatids possess a unique thiol metabolism based on trypanothione–trypanothione reductase, which plays a crucial role in regulating the redox balance and the defense against oxidative stress in a pathway not shared by the human host.[10] In the present study, we investigated the effect of four mesoionic compounds (MI-HH, MI-4-NO_2, MI-4-OCH_3 and MI-3-OCH_3) in in vitro assays as potential inhibitors of trypanothione reductase. Among the four tested compounds, only MI-4-NO_2 showed an enzyme inhibition effect on the four extracts assayed. This inhibition assay using MI-4-NO_2 was also performed on recombinant enzymes from *T. cruzi* and *L. infantum*. The best activity of MI-4-NO_2 could be related to pharmacokinetic and pharmacodynamic parameters,[43] which were not studied in this work. The enzyme kinetics determined with *Li*TryR demonstrated a non-competitive inhibition profile by MI-4-NO_2.

The four mesoionic compounds were predicted to effectively dock into the substrate binding site, independently of the species, but only in the binary complexes MI-4-NO_2 is predicted as the best ligand for the TryR of all four parasite species. Because the substrate binding site is quite large, both substrate and mesoionic compound could be also accommodated simultaneously into the cavity forming ternary complexes. However, the presence of the substrate was deleterious for the mesoionic compounds binding, as can be observed by the lowering of their respective fitness scores; besides, differently from the binary complexes, now MI-4-NO_2 was not predicted as the best ligand. MI-3-OCH_3 was predicted as the best ligand for *La*TryR and MI-4-OCH_3 for the other parasites, which is not in accordance with the experimental data.

These results are suggestive that the activity of the mesoionic compounds could be related to the binding to another TryR binding site; interestingly, recent results obtained by Bilia and co-workers indicated that naphthodianthrone inhibitors of thioredoxin

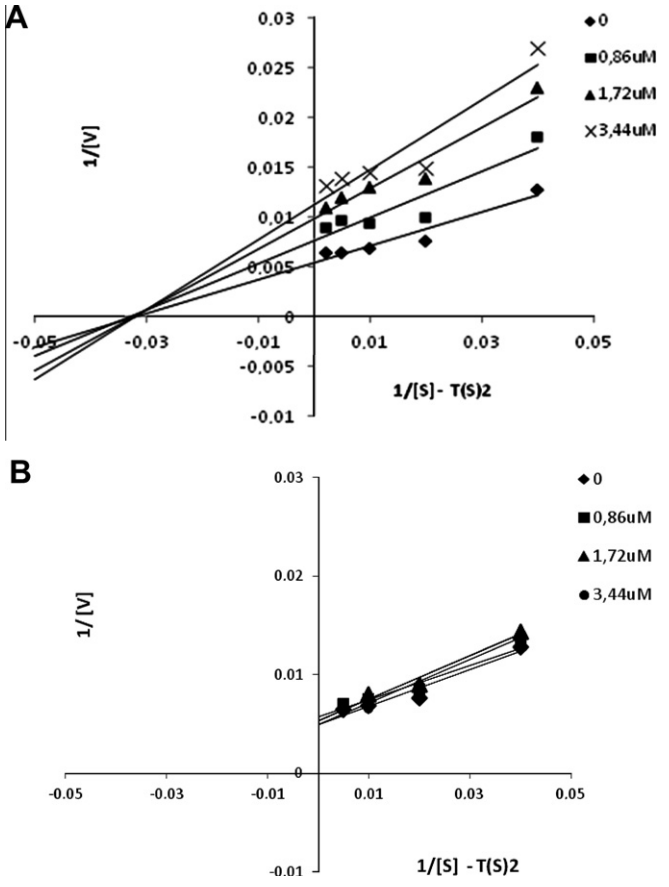


Figure 4. Kinetic analysis of *Li*TryR activity. The enzyme concentration was maintained as a constant, while substrate $[T(S)_2]$ and compounds varied, as follows: (A) MI-4-NO_2, no addition (diamonds); 0.86 μM (squares); 1.72 μM (triangles); 3.44 μM (asterisks), $IC_{50} = 1.63 \pm 0.06$; (B) MI-HH, no addition (diamonds); 0.86 μM (squares); 1.72 μM (triangles); 3.44 μM (circle), IC_{50} = not determined.

Table 1
Docking results in the TSST binding site

Ligand	Binary complexes[a]				Ternary complexes[a,b]			
	*La*TryR	*Li*TryR	*Lb*TryR	*Tc*TryR	*La*TryR	*Li*TryR	*Lb*TryR	*Tc*TryR
MI-HH	28.12	28.87	28.63	29.21	20.17	18.63	20.33	21.20
MI-3-OCH_3	29.65	30.24	32.05	32.64	*25.44*	21.12	22.15	22.91
MI-4-NO_2	*32.29*	*32.31*	*33.74*	*33.60*	23.88	20.25	*24.10*	23.09
MI-4-OCH_3	30.59	29.55	31.94	31.41	23.87	*21.77*	23.68	*24.62*
TSST	32.36	36.06	32.28	38.58				

[a] Fitness scores, ASP function.
[b] Docking of the ligands together with the substrate in the binding site.

Table 2
Docking results into the FAD and NADPH binding sites

Ligand	NADPH binding site[a,b]				FAD binding site[a,c]				FAD binding site[a,d]			
	*La*TryR	*Li*TryR	*Lb*TryR	*Tc*TryR	*La*TryR	*Li*TryR	*Lb*TryR	*Tc*TryR	*La*TryR	*Li*TryR	*Lb*TryR	*Tc*TryR
MI-HH	29.12	37.3	31.41	29.92	26.02	*34.18*	28.55	18.22	20.54	19.56	22.09	22.53
MI-3-OCH_3	30.11	34.6	32.2	29.69	26.99	29.69	30.44	13.66	20.97	20.81	22.75	22.98
MI-4-NO_2	30.67	*42.38*	*36.49*	*32.35*	*29.81*	27.81	*33.67*	*24.73*	*23.35*	*23.55*	*25.38*	*24.64*
MI-4-OCH_3	*32.47*	40.76	34.5	30.58	25.74	28.18	30.69	20.04	24.41	19.86	24.29	24.54
FAD					56.45	71.02	65.18	77.93				
NADPH	41.44	54.76	44.85	40.96								

[a] Fitness scores, ASP function.
[b] Docking of the ligands into the NADPH binding site of the FAD–TryR binary complex.
[c] Docking of the ligands into the FAD binding site of the NADPH–TryR binary complex.
[d] Docking of the ligands into the FAD binding site of the NADPH–TSST–TryR ternary complex.

reductase, a FAD-dependent oxidoreductase, bind to the NADPH binding site.[44] It can be observed in Table 2 that the ligands can dock favorably into de FAD binding site, but the presence of TSST in the adjacent binding site influenced unfavorably the docking of the ligands, as can be seen by the score values listed in Table 2. The docking of the mesoionic compounds into the NADPH site, however, was predicted as better than into the FAD site, and also than in the TSST site (Table 1). The docking results show additionally that MI-4-NO_2 is predicted as the best ligand of the NADPH binding site for three of the parasite species (it is the second best ligand for *La*TryR).

Figure 5 (A and B) presents a superimposition of the best docking poses of the mesoionic compounds inside the *Li*TryR NADPH binding site. All compounds occupy the same binding site, but with different orientations. The nitro group of MI-4-NO_2 makes a H bond with Lys60 side chain (O···N distance of 3.47 Å); the planarity of the nitro group apparently helps the *p*-nitro-phenyl group to make π–π interactions with the isoalloxazine ring of the FAD cofactor, which is responsible for its redox behavior, so it is expected that these interactions could interfere with the enzyme activity (Fig. 5B). This is not the case of the methoxy substituent, and the *p*-methoxy-phenyl and the *m*-methoxy-phenyl substituents of MI-4-OCH_3 and MI-3-OCH_3, respectively, are turned to the opposite side in the best poses identified by the docking procedure (Fig. 5A). These observations explain the best score of the MI-4-NO_2 and could be related to its experimental inhibitory activity.

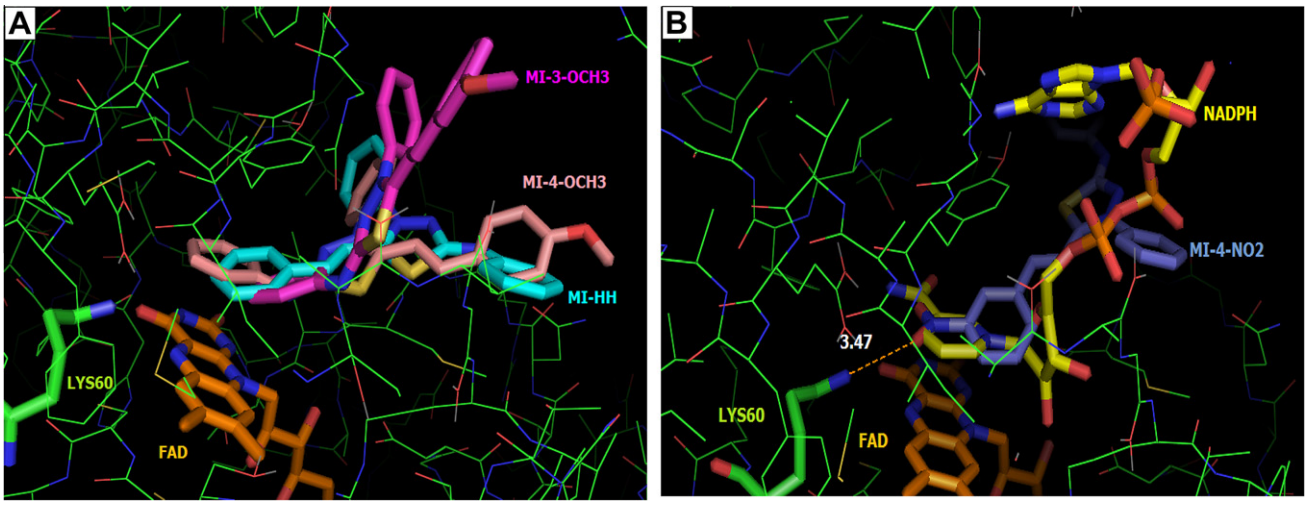


Figure 5. (A) Superposition of the best poses of the three inactive mesoionic compounds in *Li*TryR NADPH binding site. Carbon atoms: MI-3OCH_3: magenta; MI-4OCH_3: pink; MIHH: cyan; FAD: orange, TryR: green. (B) Superposition of the best poses of the active mesoionic compound and of NADPH in the *Li*TryR NADPH binding site. Carbon atoms: MI-4NO_2: purple; NADPH: yellow; FAD: orange, TryR: green. Hydrogen atoms were removed to improve clarity.

5. Conclusion

Although the triage of mesoionic compounds via the TryR assay has resulted in the identification of MI-4-NO_2 as the only active enzyme inhibitor, potent antiparasitic activities have been shown for all mesoionic compounds tested here. Regardless of the mechanism of action, the observation of potent antiparasitic activity for the mesoionic compounds is interesting and potentially therapeutically relevant. Other mechanisms of action of mesoionic derivatives, independent of TryR inhibition, remain to be established. It is possible that more than one metabolic pathway in the parasites is involved. Mesoionic compounds, similar to other compounds cited in the scientific literature, are clearly not 'magic bullets' that specifically inhibit one target[45]; instead, they take advantage of 'polypharmacology'.[46,47] The results obtained in this work will be useful for future studies of mesoionic compounds as part of a drug discovery program against Leishmaniasis or Chagas' disease.

Acknowledgments

We are grateful to the Coordenação de Aperfeicoamento de Pessoal de Nível Superior (CAPES), Conselho Nacional de Desenvolvimento Cientíıfico e Tecnológico (CNPq), Fundação de Amparo a Pesquisa do Estado do Rio de Janeiro (FAPERJ), Fundação Oswaldo Cruz (FIOCRUZ), INCT-INOFAR, Fundação para a Ciência e Tecnologia, Instituto de Biologia Molecular e Celular of Universidade do Porto and COSTDrugs2 for fellowships and financial support.

References and notes

1. World Health Organization (**2005**). Available from: http://www.who.int/tdr/diseases/chagas/swg_chagas.pdf.
2. Marin-Neto, J. A.; Rassi, A., Jr.; Avezum, A., Jr.; Mattos, A. C.; Rassi, A.; Morillo, C. A.; Sosa-Estani, S.; Yusuf, S.and BENEFIT Investigators *Mem. Inst. Oswaldo Cruz* **2009**, *10*, 319.
3. Maya, J. D.; Repetto, Y.; Agosín, M.; Ojeda, J. M.; Tellez, R.; Gaule, C.; Morello, A. *Mol. Biochem. Parasitol.* **1997**, *8*, 101.
4. Nunes, W. S.; Araújo, S. R.; Calheiros, C. M. *Braz. J. Infect. Dis.* **2010**, *1*, 342.
5. World Health Organization (**2007**). Leishmaniasis: disease information. Available from: http://www.who.int/tdr/diseases/leish/diseaseinfo.htm.
6. Croft, S. L.; Seifert, K.; Yardley, V. *Indian J. Med. Res.* **2006**, *12*, 399.
7. Carvalho, P. B.; Arribas, M. A. G.; Ferreira, E. I. *Braz. J. Pharm. Sci.* **2000**, *3*, 69.
8. Fairlamb, A. H.; Blackburn, P.; Ulrich, P.; Chait, B. T.; Cerami, A. *Science* **1985**, *22*, 1485.
9. Schmidt, A.; Krauth-Siegel, R. L. *Curr. Top. Med. Chem.* **2002**, *2*, 1239.
10. Müller, S.; Liebau, E.; Walter, R. D.; Krauth-Siegel, R. L. *Trends Parasitol.* **2003**, *1*, 320.
11. Bond, C. S.; Zhang, Y.; Berriman, M.; Cunningham, M. L.; Fairlamb, A. H.; Hunter, W. N. *Structures* **1999**, *7*, 81.
12. Newton, C. G.; Ramsden, C. A. *Tetrahedron* **1982**, *38*, 2965.
13. Moura, G. L. C. *Chem. Phys. Lett.* **1996**, *25*, 639.
14. Rakov, N.; de Araújo, C. B.; Rocha, G. B.; Simas, A. M.; Athayde-Filho, P. A.; Miller, J. *Chem. Phys. Lett.* **2000**, *33*, 13.
15. da Silva, E. F.; Canto-Cavalheiro, M. M.; Braz, V. R.; Cysne-Finkelstein, L.; Leon, L. L.; Echevarria, A. *Eur. J. Med. Chem.* **2002**, *3*, 979.
16. Rodrigues, R. F.; da Silva, E. F.; Echevarria, A.; Fajardo-Bonin, R.; Amaral, V. F.; Leon, L. L.; Canto-Cavalheiro, M. M. *Eur. J. Med. Chem.* **2007**, *4*, 1039.
17. da Silva Ferreira, W.; Freire-de-Lima, L.; Saraiva, V. B.; Alisson-Silva, F.; Mendonça-Previato, L.; Previato, J. O.; Echevarria, A.; de Lima, M. E. *Bioorg. Med. Chem.* **2008**, *1*, 2984.
18. Rodrigues, R. F.; Charret, K. S.; da Silva, E. F.; Echevarria, A.; Amaral, V. F.; Leon, L. L.; Canto-Cavalheiro, M. M. *Antimicrob. Agents Chemother.* **2009**, *5*, 839.
19. Cadena, S. M.; Carnieri, E. G.; Echevarria, A.; de Oliveira, M. B. *FEBS Lett.* **1998**, *44*, 46.
20. Grynberg, N.; Santos, A. C.; Echevarria, A. *Anticancer Drugs* **1997**, *8*, 88.
21. Senff-Ribeiro, A.; Echevarria, A.; Silva, E. F.; Sanches Veiga, S.; Oliveira, M. B. *Melanoma Res.* **2003**, *1*, 465.
22. Camargo, E. P. *Rev. Inst. Med. Trop. Sao Paulo* **1964**, *6*, 93.
23. Campos, M. C.; Salomão, K.; Castro-Pinto, D. B.; Leon, L. L.; Barbosa, H. S.; Maciel, M. A.; de Castro, S. L. *Parasitol. Res.* **2010**, *10*, 1193.
24. Castro-Pinto, D. B.; Lima, E. L.; Cunha, A. S.; Genestra, M.; De Léo, R. M.; Monteiro, F.; Leon, L. L. *J. Enz. Inhib. Med. Chem.* **2007**, *2*, 71.
25. Castro-Pinto, D. B.; Genestra, M.; Menezes, G. B.; Waghabi, M.; Gonçalves, A.; De Nigris, D. C. C.; Sant'Anna, C. M.; Leon, L. L.; Mendonça-Lima, L. *Arch. Microbiol.* **2008**, *18*, 375.
26. Gallo, M. B.; Marques, A. S.; Vieira, P. C.; da Silva, M. F.; Fernandes, J. B.; Silva, M.; Guido, R. V.; Oliva, G.; Thiemann, O. H.; Albuquerque, S.; Fairlamb, A. H. *Cham. Z. Naturforsch.* **2008**, *6*, 371.
27. Moreno, S. N.; Carnieri, E. G.; Docampo, R. *Mol. Biochem. Parasitol.* **1994**, *6*, 313.
28. Berman, H. M.; Battistuz, T.; Bhat, T. N.; Bluhm, W. F.; Bourne, P. E.; Burkhardt, K.; Feng, Z.; Gilliland, G. L.; Iype, L.; Jain, S.; Fagan, P.; Marvin, J.; Padilla, D.; Ravichandran, V.; Schneider, B.; Thanki, N.; Weissig, H.; Westbrook, J. D.; Zardecki, C. *Acta Crystallogr. D. Biol. Crystallogr.* **2002**, *5*, 899.
29. Baiocco, P.; Colotti, G.; Franceschini, S.; Ilari, A. *J. Med. Chem.* **2009**, *5*, 2603.
30. Altschul, S. F.; Madden, T. L.; Schäffer, A. A.; Zhang, J.; Zhang, Z.; Miller, W.; Lipman, D. J. *Nucleic Acids Res.* **1997**, *2*, 3389.
31. Bairoch, A.; Boeckmann, B. *Nucleic Acids Res.* **1991**, *1*, 2247.
32. Bairoch, A.; Boeckmann, B. *Nucleic Acids Res.* **1993**, *2*, 3093.
33. Guex, N.; Peitsch, M. C. *Electrophoresis* **1997**, *1*, 2714.
34. Guex, N.; Diemand, A.; Peitsch, M. C. *Trends in Biochem. Sci.* **1999**, *2*, 364.
35. W.F. van Gunsteren. Biomolecular simulations: the GROMOS96 manual and user guide. VdF Hochschulverlag ETHZ, Zürich, (**1996**).
36. Stewart, J. J. P. *J. Comp. Chem.* **1989**, *1*, 209.
37. Verdonk, M. L.; Cole, J. C.; Hartshorn, M. J. *Proteins* **2003**, *52*, 609.
38. Eldridge, M. D.; Murray, C. W.; Auton, T. R.; Paolini, G. V.; Mee, R. P. *J. Computer-Aided Mol. Design* **1997**, *1*, 425.
39. Mooij, W. T. M.; Verdonk, M. L. *Prot. Struct. Func. Bioinfor.* **2005**, *6*, 272.
40. Korb, O.; Stützle, T.; Exner, T. E. *Swarm Intelligence* **2007**, *1*, 115.
41. Korb, O.; Stützle, T.; Exner, T. E. *J. Chem. Inform. Modeling* **2009**, *4*, 84.
42. da Silva Ferreira, W.; Freire-de-Lima, L.; Saraiva, V. B.; Alisson-Silva, F.; Mendonça-Previato, L.; Previato, J. O.; Echevarria, A.; de Lima, M. E. *Bioorg. Med. Chem.* **2008**, *1*, 2984.
43. Oliveira Carlos, R. B.; Zanil, L.; Ferreira, R. S.; Leite, R. S.; Alves, T. M. A.; da Silva, T. H. A.; Romanha, A. J. *Quim. Nova* **2008**, *2*, 261.
44. Sorrentino, F.; Karioti, A.; Gratteri, P.; Rigobello, M. P.; Scutari, G.; Messori, L.; Bindoli, A.; Chioccioli, M.; Gabbiani, C.; Bergonzi, M. C.; Bilia, A. R. *Bioorg. Med. Chem.* **2011**, *1*, 631.
45. König, J.; Wyllie, S.; Wells, G.; Stevens, M. F.; Wyatt, P. G.; Fairlamb, A. H. *J. Biol. Chem.* **2011**, *28*, 8523.
46. Hopkins, A. L. *Nat. Chem. Biol.* **2008**, *4*, 682.
47. Singh, S.; Sivakumar, R. *J. Infect. Chemother.* **2004**, *1*, 307.
48. Strickland, C. L.; Puchalski, R.; Savvides, S. N.; Karplus, P. A. *Acta Crystallogr. Biol. Crystallogr.* **1995**, *3*, 334.