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RESEARCH ARTICLE

1,2,4-thiadiazol-5(4*H*)-ones: a new class of selective inhibitors of *Trypanosoma cruzi* triosephosphate isomerase. Study of the mechanism of inhibition

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Abstract

Context: Triosephosphate isomerase (TIM) is a ubiquitous enzyme that has been targeted for the discovery of small molecular weight compounds with potential use against *Trypanosoma cruzi*, the causative agent of Chagas disease. We have identified a new selective inhibitor chemotype of TIM from *T. cruzi* (*Tc*TIM), 1,2,4-thiadiazol-5(4*H*)-one.

Objective: Study the mechanism of *Tc*TIM inhibition by a 1,2,4-thiadiazol derivative.

Methods: We performed the biochemical characterization of the interaction of the 1,2,4-thiadiazol derivative with the wild-type and mutant *Tc*TIMs, using DOSY-NMR and MS experiments. Studies of *T. cruzi* growth inhibition were additionally carried out.

Results and conclusion: At low micromolar concentrations, the compound induces highly selective irreversible inactivation of *Tc*TIM through non-covalent binding. Our studies indicate that it interferes with the association of the two monomers of the dimeric enzyme. We also show that it inhibits *T. cruzi* growth in culture.

Keywords: Chagas disease, *Tc*TIM, thiadiazol, inactivation mechanism

Introduction

Trypanosoma cruzi (*T. cruzi*) has been identified for years as the causative agent of Chagas disease, an endemic disease widely disseminated in the American continent that affects 9.8–11.0 million people, leaving 60 million people at risk[1]. Currently available therapies have shown to be inadequate concerning safety, efficacy, resistance, toxicity, difficulty of administration in impoverished conditions and cost issues. Despite the urgent need for new drugs, most pharmaceutical companies have neglected this disease, largely because of market policies.

T. cruzi dependence on glycolysis makes enzymes in this pathway excellent drug targets. Particularly, triosephosphate isomerase from *Trypanosoma cruzi* (*Tc*TIM), widely investigated for the search of small molecular weight compounds that could selectively inhibit it, killing the parasite[2–4].

TIM catalyzes the isomerization of glyceraldehyde-3-phosphate and dihydroxyacetone phosphate in the fifth step of the glycolytic pathway. Most of the described TIMs are homodimers and each monomer consists of eight parallel β-strands, surrounded by eight α-helices

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Abbreviations

TIM, triosephosphate isomerase;
*Tc*TIM, triosephosphate isomerase from *Trypanosoma cruzi;*
*Tb*TIM, triosephosphate isomerase from *Trypanosoma brucei;*
*Lm*TIM, triosephosphate isomerase from *Leishmania mexicana;*
*Hs*TIM, triosephosphate isomerase from *Homo sapiens;*
DOSY-NMR, diffusion-ordered nuclear magnetic resonance experiment;
EDTA, ethylenediaminetetraacetic acid;
GAP, glyceraldehyde 3-phosphate;
αGPDH, α-glycerol phosphate dehydrogenase;
NS-nanoLC-MS, nano spray ionization liquid chromatography-mass spectrometry analysis;
PL, phospholipids;
BHI, brain–heart infusion;
ID, inhibitory dosis;
IC, inhibitory concentration;
GdnHCl, guanidine chloride;
HOMO, highest energy occupied molecular orbital

forming the classical α/β-barrel structure. In *Tc*TIM[5], the interface between monomers represents a significant portion of the molecular surface area of each polypeptide chain: approximately 1496 Å^2 in *Tc*TIM[5]. In most organisms, including *T. cruzi*, only the homodimeric form of the enzyme is active[6,7].

We examined the interface of TIM from three different trypanosomatids, *T. cruzi*, *Trypanosoma brucei* (*Tb*TIM) and *Leishmania mexicana* (*Lm*TIM) and five other species, yeast, chicken, *Homo sapiens* (*Hs*TIM), *Plasmodium falciparum* and *Entamoeba histolytica*. Our data shows that the structure of this portion of the interface is highly conserved in the enzymes from the trypanosomatids (*Tc*TIM, *Lm*TIM and *Tb*TIM), differing from the other analyzed species[8]. In fact, the identity of the 32 interfacial residues of *Tc*TIM, *Tb*TIM and *Lm*TIM with the homology of *Hs*TIM is approximately 52 %. TIMs from *T. cruzi*, *T. brucei* and *L. mexicana* have a high degree of sequence identity; being of 73.9 % for the trypanosomal TIMs, with a sequence similarity of 92.4 %[9]. In fact, the potential use of protein–protein interfaces of homodimeric enzymes as targets for drug discovery has recently been reviewed[10].

As part of our ongoing program in the search of molecules that could provide leads in the design of a new drug for the treatment of Chagas disease[8,9,11], we undertook a massive screening for inhibitors of *Tc*TIM, using nearly 230 compounds from an in-house library[12]. We identified two compounds (**1** and **2**, Figure 1), belonging to the 1,2,4-thiadiazol and 1,3,4-oxathiazol chemotypes, that displayed selectivity for *Tc*TIM over *Hs*TIM. Other 1,2,4-thiadiazol derivatives (i.e. **3** and **4**, Figure 1) exerted very low inhibition.

In this work, we characterize the mechanism through which compound **1** causes *Tc*TIM inactivation. Our data shows that it is a noncompetitive inhibitor that induces the dissociation of the two monomers and inhibits their association after they were unfolded by high guanidine chloride concentrations. In order to determine the residues or amino acids sequences of the enzyme that are essential for the action of compound **1**, we studied its effect on some *Tc*TIM mutants. We further show that, although compound **1** binds non-covalently to the enzyme, the binding character is strong as revealed by diffusion nuclear magnetic resonance (DOSY-NMR) experiments, and it is an effective inhibitor of *T. cruzi* growth.

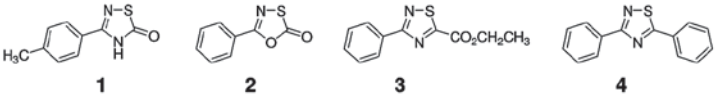
Methods

Expression and purification of proteins

*Tc*TIM[13], *Tb*TIM[14], *Lm*TIM[15], *Tc*TIM 2,3,5-8 (a chimeral protein that contains amino acids 1 to 35 and 92 to 119 of *Tb*TIM and the rest is identical to *Tc*TIM)[16] and *Tc*TIMC15A (a mutant protein, that contains an Ala-residue at position 14 of the sequence instead of a Cys present in wild-type *Tc*TIM)[17], were expressed in *Escherichia coli* and purified as described in the referred literature. After purification, the enzymes, dissolved in 100 mM triethanolamine, 10 mM EDTA and 1 mM dithiothreitol (pH 8), were precipitated with ammonium sulfate (75% saturation) and stored at 4 °C. Before use, extensive dialysis against 100 mM triethanolamine/10 mM EDTA (pH 7.4) was performed. Protein concentration was determined by absorbance at 280 nm[18].

Activity assays

Activity was determined following the conversion of glyceraldehyde 3-phosphate into dihydroxyacetone phosphate[2]. The decrease in absorbance at 340 nm was followed in a multicell Hewlett-Packard spectrophotometer at 25°C. The reaction mixture (1 mL, pH 7.4) contained 100 mM triethanolamine, 10 mM EDTA, 0.2 mM NADH, 1 mM glyceraldehyde 3-phosphate (GAP), and 0.9 units of α-glycerol phosphate dehydrogenase (α-GPDH). The reaction was initiated by addition of 5 ng/mL of the corresponding TIM. To calculate the kinetic



	1	2	3	4
TcTIM IC_{50}	3.5 μM	10.0 μM	30 μM < IC_{50} < 100 μM	IC_{50} ~ 100 μM
HsTIM IC_{50}	~ 100.0 μM	> 100.0 μM	not studied	not studied

Figure 1. Chemical structure of 1,2,4-thiadiazol derivatives and the previously studied[12] 1,3,4-oxathiazol. Derivatives **1** and **2** were also analyzed against *Hs*TIM showing at least 10-fold selectivity for *Tc*TIM.

parameters, we used increasing GAP concentrations from 0.05 to 10 mM. The data were adjusted according to the Michaelis-Menten model and the values of *K*m and Vmax were calculated by non-linear regression.

Effect of compound 1 on the stability of *Tc*TIM dimers

The enzyme was incubated at concentrations between 6×10^{-4} and 6×10^{-1} μM for 2 h at 37°C with and without of compound **1** (3.5 μM), and the remaining activity was determined.

Folding/reactivation assay

TIM (*Tc*TIM, *Hs*TIM, or *Tb*TIM, 0.5 mg/mL) was denatured with guanidine chloride (6 M) in a final volume of 200 μL for 1 h. A portion (10 μL) of this solution was diluted to a final volume of 1 mL containing increasing concentrations of compound **1**. After 15 and 60 min, 1 μL of mixture was diluted to 1 mL and the activity was determined as previously described (see Activity assays).

DOSY-NMR experiments

NMR experiments were recorded on a Varian VNMR500 (499.8 MHz) with a z-axis gradient coil. The experiments were done with a spectral window of 8012.8 Hz (14.0 to −2.0 ppm) at 298 K. Compounds **1** and **2** were dissolved in deuterated 20 mM phosphate buffer, pH 7.4, at a final dose of 11 mM in 500 μL. In the different experiments, 70 μL of *Tc*TIM (7.4 mg/mL 137 μM), prepared in the same buffer, were added to the compound solutions to reach a ratio compound:*Tc*TIM of 650:1. Diffusion experiments: For each 2D-DOSY experiment 32 acquisitions were carried out, in which the gradient strength G went from 1.83 to 40.3 G cm^{-1}, the diffusion time was 50 ms and the gradient pulse time 2 ms. Each experiment was 36 min long and the signal of H_2O was suppressed by presaturation.

Mass spectrometry analysis

For the molecular mass determinations of the whole enzyme, native and TIM treated with compound **1** (using the same conditions used for enzyme activity assays) were bound on a C(4) Ziptip (P10, Millipore Corp., Bedford, MA), washed with H_2O-0.1% formic acid and directly eluted into the ESI-ion trap with 30% AcCN and 0.1% formic acid[19]. The deconvoluted spectra were obtained by using the Promass Deconvolution for Xcalibur Software (Thermo Electron Corp.).

Peptide mapping and nanospray ionization liquid chromatography-mass spectrometry analysis

Native and TIM treated with compound **1** (1 μmol) were digested with sequencing grade trypsin in 50 mM bicarbonate buffer, pH 8.0 at 37 °C for 15 h using an enzyme:substrate ratio of approximately 1:50 (w/w). In some cases, protein was reduced with dithiothreitol (25 mM) and alkylated with iodoacetamide (100 mM) before digestion. Peptide samples were analyzed by nanospray ionization liquid chromatography-mass spectrometry analysis (NSI-nanoLC-MS) using a LTQ Velos ion trap mass spectrometer (Thermo Electron Corp., San Jose, CA) in line with an Easy nLC (formerly Proxeon, Thermo Electron Corp., San Jose, CA). A reversed phase pre-column (EASY-Column, 2 cm, ID100 μm, 5 μm) in line with an analytical reverse phase column (EASY-Column, 10 cm, ID 75 μm, 3 μm) from Thermo (Thermo Electron Corp., San Jose, CA) were used at 300 nL/min, using a gradient from 0–45% of solvent B in 70 min (solvent A: 0.1% formic acid; solvent B: acetonitrile, 0.1% formic acid). The nanospray voltage was 1.4 kV, and the temperature of the capillary was 230°C. Peptides were detected in the positive ion mode using a mass range of 300–2000 m/z. The top 5 MS/MS peptide analyses were performed by data dependent acquisition. The data was analyzed by using Proteome Discoverer software (Thermo Electron Corp., San Jose, CA), using Sequest and/or Mascot as search engines, and allowing the following dynamic modifications: dehydro-Cys, carboxamidomethyl-Cys, Met oxidation.

Theoretical calculations

The molecular structures of compounds **1–4**, were subjected to complete geometry optimization, in gas phase, using the PC SPARTAN 04 package[20] as follows: Conformational search using the MMFF conformer module, the structure of the most stable conformer was optimized by applying density functional B3LYP-6-31G*. Structural, geometrical and electronic descriptors were extracted from comparisons.

Anti-*T. cruzi* activity of compound 1 encapsulated in liposomes

The liposomes were prepared by hydrating thin lipid films by shaking them with biological culture milieu at room temperature. The lipid films were obtained by evaporation at reduced pressure in a rotary evaporator[21], from a chloroformic solution of phospholipid (PL) (P3556L-α-Phosphatidylcholine (100 mg), Aldrich) containing different concentrations of compound **1**. The biological evaluation was performed as previously described[22]. Briefly, *T. cruzi* epimastigotes (Tulahuen 2 strain) were grown axenically at 28°C in BHI-Tryptose, complemented with 5% fetal calf serum. Cells were harvested in the late log phase, suspended in fresh culture medium, counted in a Neubauer chamber and placed in 24-well plates (2×10^6 cells/mL). Cell growth was measured by counting trypanosomes in the Neubauer chamber. Before inoculation, the medium was supplemented with the indicated amount of liposomes containing the compound to be analyzed. No effect on the growth of epimastigotes was observed at the maximum concentration of PL (1000 μM) without compound. The percent of growth inhibition was calculated as follows: {1 – [(Ap – A0p) / (Ac – A0c)]} × 100, where Ap = amount of parasites containing the compound to be analyzed on day 5; A0p = amount of parasites containing the compound to be analyzed, just after adding the compound (day 0); Ac = amount of parasites in the

absence of compound (control) on day 5; A0c = amount of parasites in the absence of compound on day 0. To determine the ID_{50}, parasite growth was followed in the absence (control), and the presence, of increasing concentrations of the corresponding compound. The IC_{50} values were considered as the drug concentrations required to decrease the number of parasites by half of those measured for untreated controls. Nifurtimox (**Nfx**) and Benznidazole (**Bnz**) were used as reference drugs.

Results

Effect of compounds 1 and 2 on the activity of different TIMs

We studied the effect of different concentrations of compounds **1** and **2** on the activity of *Tc*TIM. The results show that although both compounds are effective inhibitors of *Tc*TIM (Table 1), compound **1** (with an IC_{50} of 3.5 μM) is about three-times more effective than compound **2**. The effect of different concentrations of compound **1** on the activity of *Tc*TIM is shown in Figure 2. It is important to denote that the activity of *Hs*TIM is not affected by 100 μM of compound **2**, and that the IC_{50} of compound **1** on the activity of *Hs*TIM is around 100 μM (Table 1). To further examine the selectivity of these compounds for *Tc*TIM, we assessed their effect on the activity of *Lm*TIM and *Tb*TIM. Our data shows that compounds **1** and **2**, at a concentration of 100 μM, induced a rather small inhibition of the latter enzymes (Table 1). Thus, the compounds seem to be highly selective for *Tc*TIM.

The kinetic studies of *Tc*TIM with and without compound **1**, using different concentrations of glyceraldehyde 3-phosphate, indicated that this compound is a noncompetitive inhibitor of the enzyme. At a concentration of 3.5 μM, compound **1** reduced the Vmax six-fold without significantly affecting the Km (Figure 2a).

Some of the most powerful inhibitors of *Tc*TIM act by lowering the association between the two monomers of the enzyme[23]. Accordingly, we explored if compound **1** exerts its action through the perturbation of monomer-monomer association. *Tc*TIM was incubated with different concentrations of the compound for 2 h and the activity was measured (Figure 2b). The data shown in Figure 3a suggest that compound **1** exerts its action by favouring the dissociation over the association reaction of TIM monomers; the presence of compound **1** increased 15-fold the concentration of TIM needed to measure the same enzyme activity detected in the absence of the compound.

To examine if the disruption of the dimeric form of TIM is due to the association of compound **1** with its monomers, *Tc*TIM was denatured in 6 M guanidine chloride (GdnHCl). In a second step of the experiment, the denaturing mixture was diluted in buffer containing different concentrations of the compound. As previously shown by[24], the removal of the denaturant by dilution leads to the refolding and association of the monomers to form the catalytically active dimer. This reaction can be followed by measuring the appearance of activity through time. Figure 3b shows that compound **1** effectively inhibits the formation of active *Tc*TIM dimers from its corresponding unfolded monomers. The IC_{50} of compound **1** for the inhibition of dimer formation was 0.6 μM and the IC_{90} was 3.0 μM.

Table 1. Classic inhibition assay of *Tc, Hs, Lm* and *Tb* TIMs to evaluate the selectivity of the inhibitor compounds.

	Inhibition			
	IC_{50} (μM)		% of inhibition at 100 μM	
Compound	*Tc*TIM	*Hs*TIM	*Lm*TIM	*Tb*TIM
1	3.5 ± 1[a] (6.0)[b]	~100[a] (59.0)[c]	2.0	16.0
2	10 ± 1[a] (200.0)[b]	>100[a] (0.0)[c]	0.0	0.0

[a]From the reference Alvarez et al.[12].
[b]The values in parentheses correspond to the IC_{90} (μM).
[c]The values in parenthesis correspond to the percentage of inhibition of enzyme activity at 100 μM.

NMR experiments

The proton signals for compounds **1** and **2** incubated with, or without, *Tc*TIM (ratio compound:*Tc*TIM=260:1) displayed the same diffusion coefficient values in both conditions. The results obtained in this experiment suggest that these compounds could be establishing covalent or strong interactions with *Tc*TIM, preventing us to evidence any weak compound-biomolecule interaction. In this conditions, compounds **1** and **2**, strongly bound to *Tc*TIM, displayed a diffusion coefficient similar to the protein alone and the fact that the new entity was at a lower concentration (free compound:bound compound = 650:1) led to the absence of new signals.

Mass spectrometry analysis

Native TIM and TIM treated with compound **1** were analyzed by mass spectrometry (see Materials and Methods) to ascertain if adducts of the inhibitor and the enzyme were formed. The deconvoluted spectra of native *versus* treated enzyme, in the same experimental conditions where inhibition was observed, showed no differences in the molecular mass of the enzyme. These results strongly suggest that the inhibitor is not forming a covalent bond with the enzyme (data not shown). To confirm the observation, we analyzed the peptide map of native *versus* treated *Tc*TIM. In both cases, 98% of the sequence was covered, including all the Cys residues. No evidence of covalent binding of the inhibitor to *Tc*TIM was detected (data not shown).

Theoretical calculations

Theoretical calculations were made in order to assess the contribution of the electrophilic centre of compound **1** to the inactivation of *Tc*TIM; its molecular similarity to the less active or inert compounds **2**–**4** was also determined. Compound **1** possesses a very different electronic distribution when compared to derivatives **2**–**4** (Figure 4); its nitrogen 2 (N2) is more negative and, consequently, may

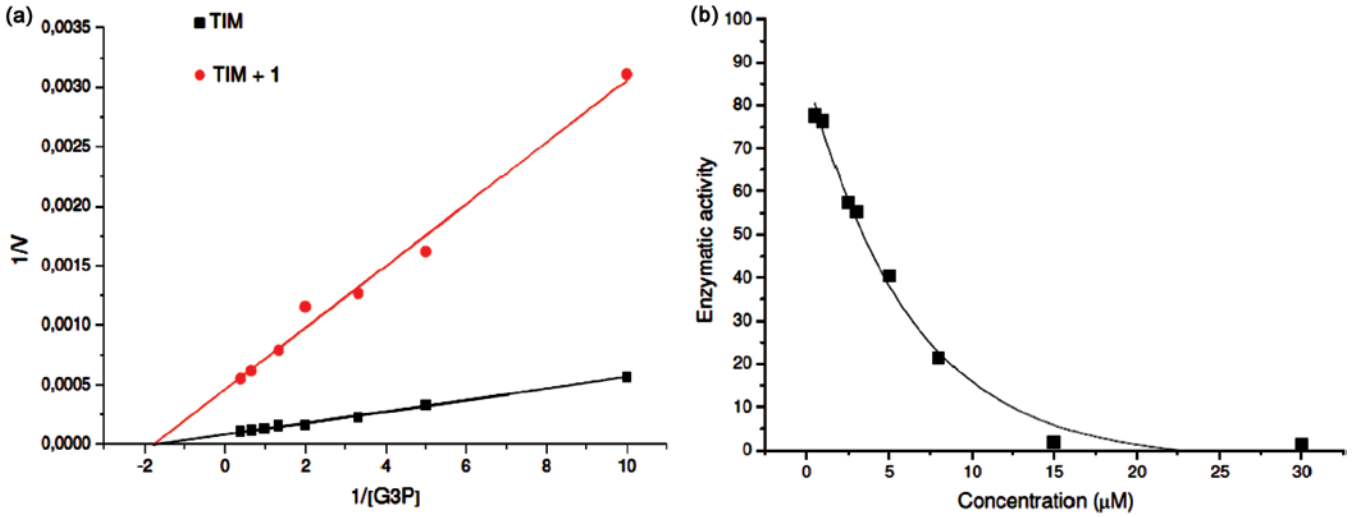


Figure 2. Effect of compounds **1** on the activity of *Tc*TIM. (a) Lineweaver–Burk plot of *Tc*TIM alone and *Tc*TIM+compound **1** (3.5 μM). (b) *Tc*TIM activity dependence on compound **1** concentration.

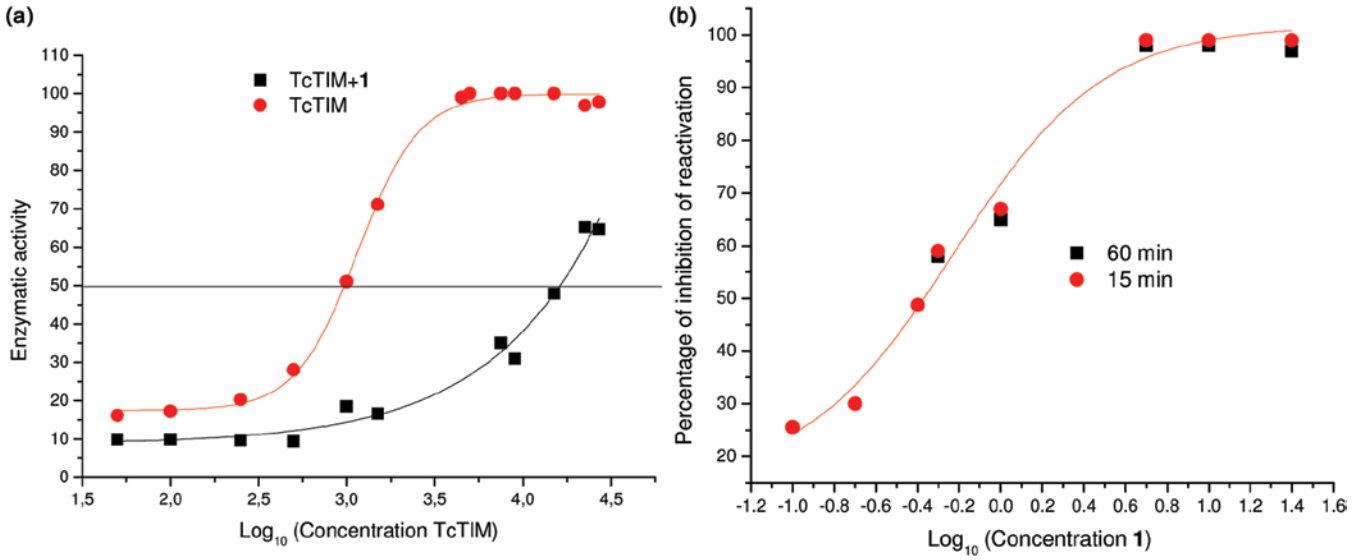


Figure 3. Effect of compound **1** on *Tc*TIM stability. (a) Effect of compound **1** on *Tc*TIM stability. The enzyme was incubated at the indicated concentrations for 2 h at 37°C with (▪) and without (●) 3.5 μM compound **1** and the activity of each of the samples was measured. (b) Effect of compound **1** on the formation of active *Tc*TIM from GdnHCl unfolded monomers. The experiment was performed as described in the Methods section. Activity was measured 15 (●) and 60 (▪) min after the denaturing mixture was diluted one hundred fold.

undergo protonation, promoting a heterocyclic opening process that may be related to its inactivating properties. Furthermore, the higher S1-N2 bond length for compound **1** (Figure 4b) clearly reinforced the idea that this compound possesses the lowest bond order and consequently the highest capability to be opened by a biological nucleophile in the bond S1-N2. Additionally, we studied the covalent interaction between compound **1** and a model of low molecular mass nucleophile-thiol, β-mercaptoethanol, in order to probe these theoretical observations (Figure S1, Supplementary Material). The mass spectrometry analysis confirmed the reactive site (S1-N2) for compound **1** (Figures S2 and S3, Supplementary Material).

We also analyzed compounds **1–4**, considering their stereo-electronic convergences, using the structural similarities-module implemented in SPARTAN package. The two more effective inhibitors, compounds **1** and **2**, share common structural features and differ in some stereo-electronic features compared to the inactive thiadiazol molecules, compounds **3** and **4** (Figures 4 and 5 for details). The good inhibitors, **1** and **2**, share the presence of a ring aromatic system (1,2,4-thiadiazol-5(4*H*)-one and 1,3,4-oxathiazol-2-one, red region, RING_AROM in Figure 5) with different stereo-electronic features than the inactive derivatives (**3** and **4**, absence of these red regions, Figure 5). Additionally, the weak inhibitors share the presence of a hydrophobic substituent, an ethyl group in compound **3** and a phenyl moiety in compound **4** (blue regions, HPHOBE and HPHOBE RING_AROM,

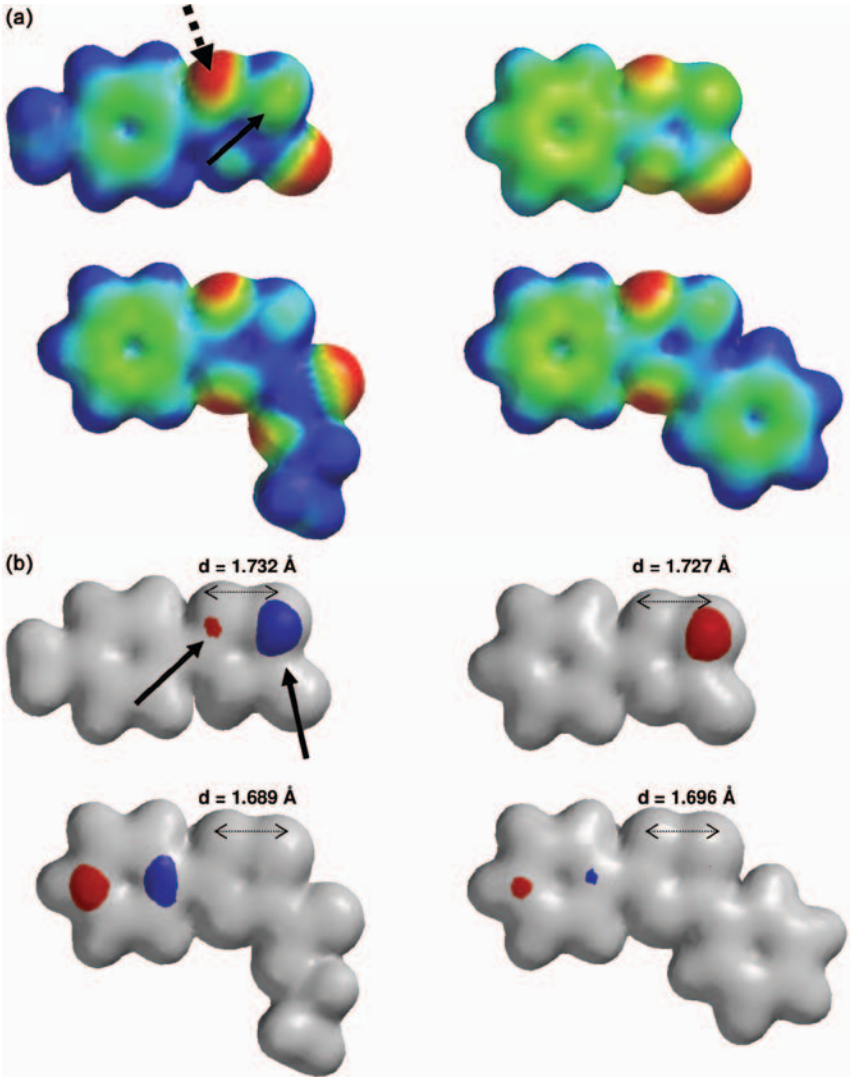


Figure 4. Calculated electronic distribution for compounds **1–4**. (a) Potential electrostatic molecular maps. The solid arrow shows the reaction centre (sulfur 1, S1) and the dotted arrow points the protonated centre (nitrogen 2, N2). (b) HOMO maps (red-blue) superimposed to electron density bond maps (silver). The solid arrows show the contribution of S1 and N2 to the HOMO only in derivative **1**. The S1-N2 bond lengths for each compound are also shown.

respectively, Figure 5) that are not present in the structures of compounds **1** and **2**. Both groups of structural characteristics could contribute to the adequate accessibility to *Tc*TIM explaining the marked differences in the potency of compounds **1**, **2**, **3** and **4** in the inactivation of *Tc*TIM.

Attempts to identify the amino acid or regions of *Tc*TIM that make it susceptible to the action of compound 1

It has been reported that the integrity of the interface Cys of *Tc*TIM is central in maintaining the stability of dimeric *Tc*TIM[10]. Therefore, we examined the effect of compound **1** on a C15A mutant of *Tc*TIM (Table 2). The mutant was inactivated by compound **1** and was slightly more sensitive than the wild type. Recently, we reported[16] that it is possible to use chimeras of *Tc*TIM and *Tb*TIM in order to identify the amino acids or amino acid sequences that are involved in a given property of the enzymes. Thus, we examined the effect of compound **1** on a *Tc*TIM in which regions 1 (residues 1–35) and 4 (residues 92–119) were replaced by the corresponding regions of *Tb*TIM, namely *Tc*TIM 2,3,5-8 (Figure 6). The chimera showed to be largely insensitive to both compounds **1** and **2** (Table 2).

Proof of concept

In order to test the hypothesis that *Tc*TIM inhibition could affect the life of *T. cruzi*, we examined the effect of compound **1** on epimastigotes in culture. It is noted that we have previously evaluated the effect of compound **1** on epimastigotes[12], and observed that it displayed very low activity against the whole parasite. We hypothesized that the poor effect of compound **1** could be explained, by its relatively high hydrophilicity that could be affecting its penetration into the parasite. Accordingly, we encapsulated compound **1** into liposomes with different proportions of phospholipids (PL), and tested their

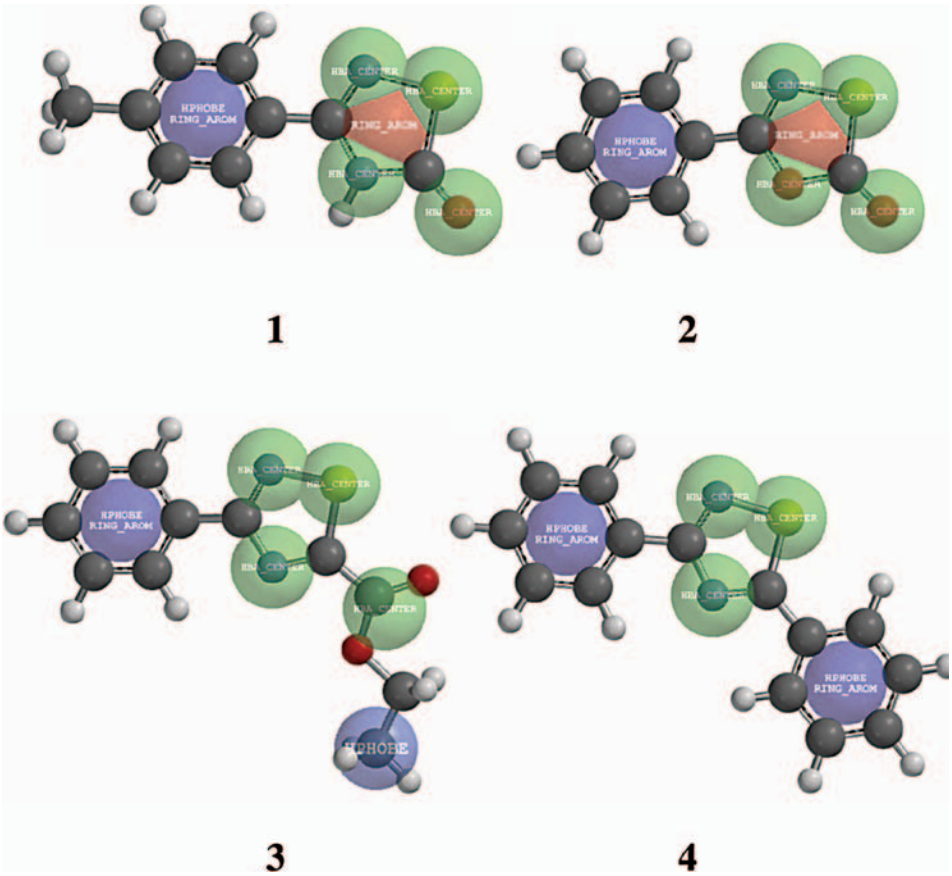


Figure 5. Structural similarities of the analyzed compounds (**1–4**).

Table 2. Classic inhibition assay, using modified *Tc*TIMs, to evaluate the selectivity of the inhibitor **1**.

	Inhibition	
	IC_{50} (μM)	% of inhibition at 100 μM
Compound	*Tc*TIMC15A	*Tc*TIM 2,3,5-8 (chimera)
1	1.5 ± 1	19
2	ns[a]	4

[a]ns, not studied.

effect on *T. cruzi* epimastigotes (Tulahuen 2 strain). In these conditions, compound **1** was an excellent inhibitor of growth for *T. cruzi* (Figure 7) with an IC_{50} of 5.8 μM, similar to the value found for inhibition of *Tc*TIM activity *in vitro*.

Discussion

Using a massive screening assay that monitors the inactivation of *Tc*TIM, we identified the 1,2,4-thiadiazol derivative **1** as a small molecule with potential anti-*T. cruzi* activity[12]. We found that this compound, together with its oxa-analogue compound **2**, selectively inhibits the *T. cruzi* enzyme, with rather meager effects on TIMs from *H. sapiens*, *Leishmania sp.* and *T. brucei*. As shown here, compound **1** diminishes the stability of the *Tc*TIM dimer and prevents its formation from unfolded monomers; furthermore, our data indicates

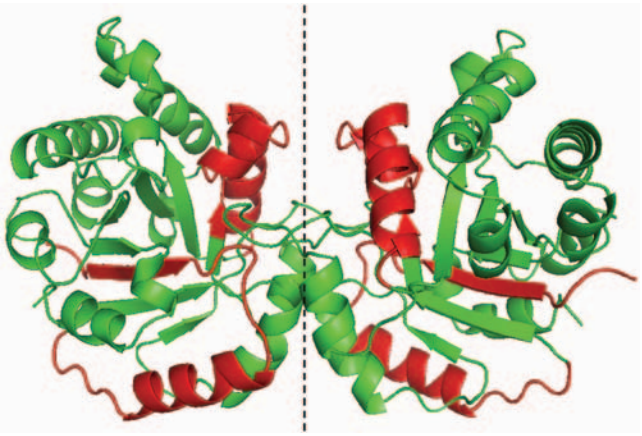


Figure 6. Stereoview of *Tc*TIM showing the regions of *Tb*TIM (in red) that were grafted in *Tc*TIM to generate the chimera studied herein.

that the inactivating effect of compound **1** on dimeric *Tc*TIM is highly specific. Nonetheless, at submicromolar concentrations, compound **1** inhibits the formation of *Tb*TIM and *Hs*TIM dimers from their respective unfolded monomers (not shown), suggesting that all the tested TIMs have a binding site for compound **1**. However, in *Tc*TIM the union site for the compound seems to be accessible in the whole dimer, whereas in the other TIMs,

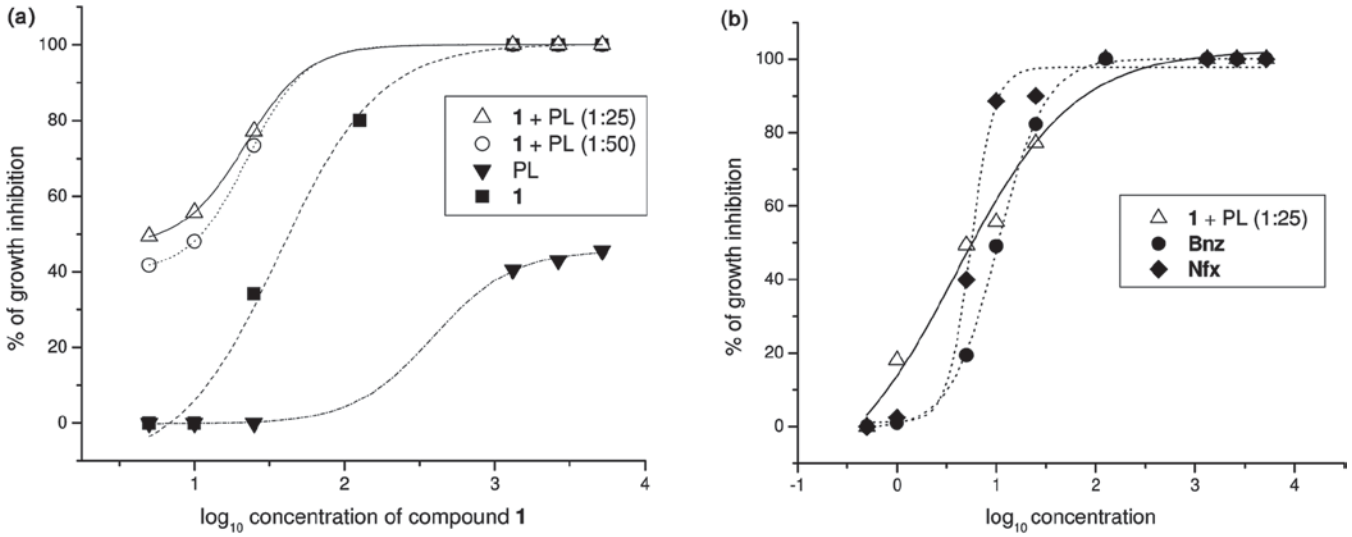


Figure 7. *T. cruzi* growth inhibition assays. (a) Dose-response curves showing growth inhibition of *T. cruzi* by compound **1** incorporated into phospholipid (PL). (b) Dose-response curves showing growth inhibition of *T. cruzi* by compound 1 incorporated into PL and the reference drugs, Nifurtimox (**Nfx**) and Benznidazole (**Bnz**).

compound **1** is able to bind to the inhibitory site only when the dimeric structure is exposed to the solvent. Thus, it would appear that the high selectivity of *Tc*TIM to compound **1** is related to a higher solvent accessibility of the binding site for the molecule. It is important to denote that the DOSY-NMR experiments of compounds **1** and **2** after incubation with *Tc*TIM (ratio compound:*Tc*TIM = 650:1), indicated that these compounds establish strong interactions with the protein. However, it is important to consider that we could not detect covalent interactions between the enzyme and compounds **1** and **2**, even though these may function as nucleophilic acceptors with the potential to bind covalently to some amino acid residues. Since we have not been able to obtain crystals of *Tc*TIM in complex with compound **1**, we could not elucidate its precise binding site. However, the data with the chimeras have given insight into the residues that contribute to the binding of the compounds to *Tc*TIM.

In summary, we have found a compound that, in the low micromolar range, specifically inhibits the activity of *Tc*TIM and inhibits *T. cruzi* epimastigotes growth.

Declaration of interest

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