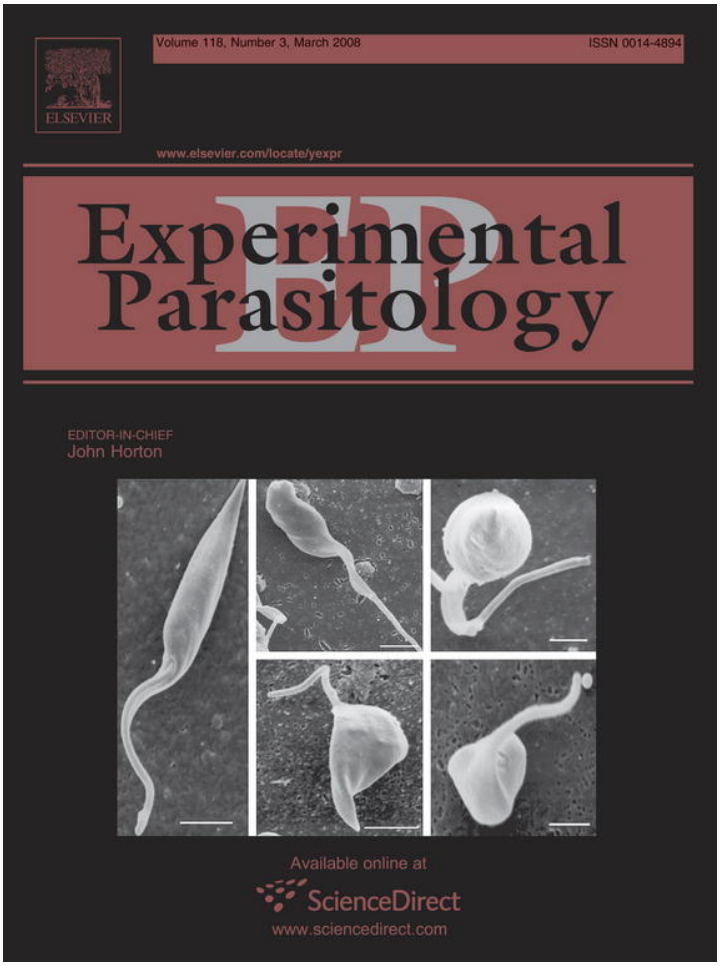

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Research brief

Trypanosoma cruzi: High ribosomal resistance to trichosanthin inactivation

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Abstract

Trypanosoma cruzi is the parasite causing Chagas Disease. Several results already published suggest that *T. cruzi* ribosomes have remarkable differences with their mammalian counterparts. In the present work, we showed that trypanosomatid (*T. cruzi* and *Crithidia fasciculata*) ribosomes are highly resistant to inactivation by trichosanthin (TCS), which is active against mammalian ribosomes. Differential resistance is an intrinsic feature of the ribosomal particles, as demonstrated by using assays where the only variable was the ribosomes source. Because we have recently described that TCS interacts with the acidic C-terminal end of mammalian ribosomal P proteins, we assayed the effect of a TCS variant, which is unable to interact with P proteins, on trypanosomatid ribosomes. This mutant showed similar shifting of IC_{50} values on rat, *T. cruzi* and *C. fasciculata* ribosomes, suggesting that the resistance mechanism might involve other ribosomal components rather than the C-terminal end of P proteins.

Index Descriptors and Abbreviations: Trypanosoma; Chagas Disease; Protein synthesis; Trichosanthin; Ribosome-inactivating protein (RIP)

1. Introduction

Trypanosoma cruzi is a protozoan kinetoplastid parasite responsible for Chagas Disease. No vaccines are available at present, and drugs used for treatment show undesirably side effects. The identification of new targets for chemotherapy is a major challenge in the control of this disease.

Recently, cryo EM has been used to elucidate the structure of the 80S *T. cruzi* ribosome (Gao et al., 2005). The corresponding density map shows a phylogenetically conserved rRNA core structure as well as distinctive structural characteristics in both the small and large subunits. These observations suggested that *T. cruzi* ribosomes have specific features that could be exploited for the development of anti-parasitic drugs. However, up to date, there are scarce reports showing differences in the protein synthesis process between mammals and trypanosomatids. An early report has shown that *T. brucei*, the parasite causing sleeping sickness, is highly resistant to ricin; a type II ribosome-inactivating protein (RIP) (Scory and Steverding, 1997). These data suggested us that other RIPs could be valuable for detecting structural and functional differences between trypanosomatid and mammalian ribosomes.

Trichosanthin (TCS) is a type I RIP, and was initially isolated from the root tuber of *Trichosanthes kirilowii*. It has been used clinically to treat hydatidiform moles, trophoblastic carcinomas, ectopic pregnancies and to interrupt early and mid-trimester pregnancies (Shaw et al., 2005). TCS, like its homolog ricin A-chain, inactivates ribosome through its RNA *N*-glycosidase activity that depurinates an invariant adenine residue, A4324 (numbering according to the rat sequence), in the conserved sarcin/ricin loop (SRL) of 28S rRNA in eukaryotic ribosomes (Endo and Tsurugi, 1987, 1988; Endo et al., 1987; Stirpe et al., 1988; Zhang and Liu, 1992). Such modification prevents binding of elongation factors to the SRL, and leads

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to the arrest of protein synthesis (Nilsson and Nygard, 1986; Sperti et al., 1973).

Although RIPs are able to cleave naked rRNA, the k_{cat} of this reaction is 10^5-fold lower than that for rRNA within an intact ribosome (Endo and Tsurugi, 1988). This finding strongly suggests that ribosomal proteins are involved in rendering the rRNA susceptible to inactivation by RIP. In an early study, ricin A-chain was cross-linked to ribosomal proteins L9 and P0 of mammalian ribosome (Vater et al., 1995). Pokeweed antiviral protein, a type I RIP, was found to interact with yeast ribosomal protein L3 (Hudak et al., 1999; Rajamohan et al., 2001). It has recently been shown that TCS interacts with L10a and the conserved C-terminal end of the acidic ribosomal proteins P0, P1 and P2 from rat (Chan et al., 2001, 2007; Xia et al., 2005).

In the present work, we explored the effect of TCS on the *in vitro* translation activity of rat, *Crithidia fasciculata* (a non-pathogenic trypanosomatid) and *T. cruzi* ribosomes. In order to avoid artifacts we performed all the assays under the same experimental conditions, being the ribosome source the only variable among different experiments.

2. Materials and methods

2.1. Expression and purification of wtTCS and K173A/R174A/K177A TCS

Both wt and triple alanine variants of TCS were expressed in *Escherichia coli* and purified as recently described (Chan et al., 2007).

2.2. Rat liver ribosome purification

All steps were performed at 4 °C. An S_{150} fraction was previously obtained by centrifugation of a rat liver homogenate (obtained as described below for ribosome purification) for 10 min at 17,000*g*. The supernatant fluid was centrifuged at 150,000*g* for 2 h. The upper 4/5 volume was carefully collected, aliquoted and stored at −80 °C until use.

For the ribosome purification, rat liver (≈20 g) was washed in sucrose 0.25 M and then homogenized in buffer containing 50 mM Tris–HCl, pH 7.5; 250 mM KCl; 5 mM magnesium acetate; sucrose 0.25 M and supplemented with 10% of S_{150} fraction (obtained as described above). The homogenate was treated with 10 U/ml of α-amylase and 0.1 mM $CaCl_2$ for 15 min at 4 °C, and then centrifuged for 4 min at low speed. The supernatant was again centrifuged for 20 min at 23,000*g*. The pellet was discarded and the supernatant containing polysomes was supplemented with Triton X-100 1%, deoxycholate 0.5% and centrifuged for 5 min at 16,000*g*. The new supernatant fluid (∼12 ml) was layered onto a discontinuous gradient of 2 M (∼6 ml) and 1.5 M (∼4 ml) sucrose made up in *Buffer A* (50 mM Tris–HCl, pH 7.5; 5 mM magnesium acetate; 250 mM KCl, dithiothreitol 1 mM and 10% of S_{150} fraction) and centrifuged 16 h at 140,000*g*. The pellet corresponding to purified ribosomes was rinsed and resuspended in a buffer containing 10 mM Tris–HCl, pH 7.5; 10 mM KCl and 1.5 mM magnesium acetate. Ribosome concentration was determined by optical density at 260 nm.

2.3. Purification of ribosomes from T. cruzi and C. fasciculata

Epimastigotes of *T. cruzi*, strain CL Brener and *C. fasciculata* (ATCC 11745) were grown up to a cell density of $3–4 \times 10^7$ cells/ml at 28 °C in rich medium containing brain heart infusion and tryptose supplemented with 10% of heat inactivated fetal bovine serum. Cycloheximide (50 μg/ml) was added 10 min before harvesting by centrifugation at 4 °C. Cells were washed twice with PBS containing 50 μg/ml cycloheximide. The cells were resuspended in *Lysis Buffer* (20 mM Tris–HCl, pH 7.5; 1 mM $MgCl_2$; 5 mM KCl; 3 mM $CaCl_2$; 5 mM 2-merchaptoethanol and 250 mM sucrose) and lysed with 0.2–0.4% of Nonidet P40 at 0 °C. The homogenate was centrifuged 2–3 times for 20 min at 12,000*g* and the final supernatant fraction containing ribosomes was layered onto a discontinuous gradient of 2 and 1.5 M of sucrose made up in the following buffer: 10 mM Tris–HCl, pH 7.5; 1 mM magnesium acetate; and 100 mM potasium acetate. The gradient was centrifuged for 16 h at 140,000*g*. The supernatant was discarded and the pellet carefully rinsed with a small volume of the same buffer without sucrose, and finally resuspended in the same buffer, aliquoted and kept frozen at −80 °C. Ribosome concentration was determined by optical density at 260 nm.

2.4. In vitro protein synthesis

The reaction mixtures were prepared on ice and contained: 19 amino acids 50 μM each (excepting Met); 2 mM dithiothreitol; 100 mM potassium acetate; 3.5 mM magnesium acetate; 75 μg/ml wheat germ tRNA; 18 mM Hepes/KOH, pH 7.5; 1 mM ATP; 0.5 mM GTP; 7.5 mM creatine phosphate; 37.5 μg/ml creatine phosphokinase; rat liver S_{150} fraction (24 μg of protein); 0.3 A_{260} U of *T. cruzi*, *C. fasciculata* or rat ribosomes and 2 μCi of ^{35}S-methionine in a final volume of 30 μl. Reactions were performed at 30 °C during 60 min and stopped by adding 150 μl of 1.5 M NaOH; 1 mM Met, 170 μg/ml BSA. After incubation for 30 min at 37 °C, proteins were precipitated with 1 ml of cold TCA 25%. After 60 min on ice, the samples were filtered and washed with TCA 10% and ethanol using glass fiber filters. Radioactivity retained in the filters was measured by liquid scintillation counting. The typical cpm control values for each system were: *T. cruzi*, 12,000 cpm; rat, 50,000 cpm and *C. fasciculata*, 58,000 cpm.

In vitro protein synthesis assays with reticulocyte lysates were performed using the TNT® Quick Coupled Transcription/Translation System (Promega) and a luciferase-encoding plasmid following the manufacturer's instructions. All data were expressed as percentage of the radioactivity

incorporated into TCA-insoluble material corresponding to control reactions in the absence of added inhibitors.

2.5. Trypanosoma cruzi in vivo protein synthesis assay

In order to evaluate the effect of hippuristanol *in vivo*, 10^7 parasites in 100 µl of PBS buffer supplemented with glucose 10 mM were incubated for 15 min at 30 °C in the presence or absence of inhibitor. After adding 0.1 µCi of ^{35}S-methionine, mixtures were further incubated for 60 min. Reactions were stopped and samples treated as described for the *in vitro* protein synthesis assays.

2.6. Statistical analysis

The significance of the protein synthesis inhibition by inhibitors and TCS was analyzed with a Student's *t* test. Values of $p < 0.05$ were considered significant. Dose–response curves and IC_{50} values were obtained by fitting the data to a four-parameter logistic equation using non-linear regression. In all the cases, the R^2 values were >0.95.

3. Results and discussion

3.1. Characterization of in vitro translation systems

In order to identify functional differences among mammalian and trypanosomatid ribosomes, we have purified ribosomes from three different sources (rat liver and the kinetoplastids *T. cruzi* and *C. fasciculata*) as described in Section 2. These ribosomes were used to perform cell-free protein synthesis assays with endogenous mRNA. The rat and *T. cruzi* ribosomal preparations have no detectable protein synthesis activity in the absence of rat liver S_{150} fraction (Fig. 1a). In contrast, the ribosomal fraction from *C. fasciculata* is able to perform protein synthesis in the absence of added supernatant factors, suggesting that the ribosomal pellet retains significant amounts of elongation factors (Fig. 1a). Moreover, the addition of rat liver supernatant to *C. fasciculata* ribosomes produced a significant ($p < 0.05$) inhibition of activity. The soluble fraction of *T. cruzi* could not be used as source of soluble factors because it contains a protein synthesis inhibitor (not shown). Similar inhibitor activities have been described in *Leishmania mexicana* (Fastame and Algranati, 1998) and *T. brucei* (Duszenko et al., 1999).

Our *in vitro* protein synthesis systems have polypeptide elongating activity, as determined by the significant ($p < 0.05$) inhibition of radioactive incorporation in the presence of emetine (Fig. 1a). In contrast, addition of 100 µM hippuristanol, a recently characterized specific inhibitor of the eukaryotic initiation factor 4A (eIF4A) (Bordeleau et al., 2006), did not affect the amino acid radioactive incorporation by *C. fasciculata*, *T. cruzi* and rat ribosomal preparations, showing that these systems were not able to initiate the protein synthesis *de novo* (Fig. 1b). It should be noted that hippuristanol strongly inhibits *T. cruzi* protein synthesis *in vivo*, as well as the reticulocyte protein synthesis system added with exogenous mRNA, where initiation effectively takes place (Fig. 1b and c). Because TCS blocks protein synthesis at the elongation step, our systems are adequate for comparing the relative resistance of different ribosomes to this RIP.

3.2. Trypanosoma cruzi and C. fasciculata ribosomes are resistant to TCS

An early work has reported that the *in vitro* protein synthesis by *T. brucei* extract is remarkably resistant to inactivation by ricin A-chain (Scory and Steverding, 1997). We have performed similar assays with TCS and observed that whereas 10 nM of toxin completely abolished the activity of the reticulocyte system, 100 nM of TCS had no effect on the activity of *T. cruzi* ribosomes (Fig. 2a). Next, we decided to use exactly the same reaction conditions (mixture reaction, ribosome concentration, etc) to analyze pro-

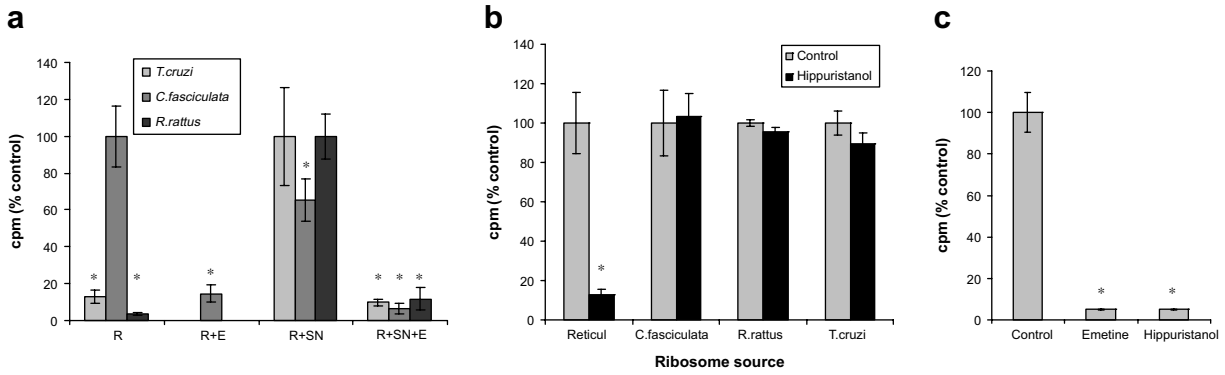

Fig. 1. (a) *In vitro* translation assays using ribosomes (R) from three different sources were performed in the presence or absence of rat liver supernatant fraction (SN) and/or the elongation inhibitor emetine (E). Optimal conditions for each ribosome preparation (with S_{150} for rat and *T. cruzi* and without S_{150} for *C. fasciculata*) were considered 100%. (b) *In vitro* translation reactions with different ribosome preparations were performed in the presence or absence of hippuristanol. As a positive control the reticulocyte lysate system was included. (c) Inhibition of protein synthesis *in vivo* in *T. cruzi* by hippuristanol (1 µM). Negative and positive controls were incubated without inhibitor and with emetine 0.1 mg/ml, respectively. All data are presented as media ± SD and asterisks indicate significant differences with control ($p < 0.05$).

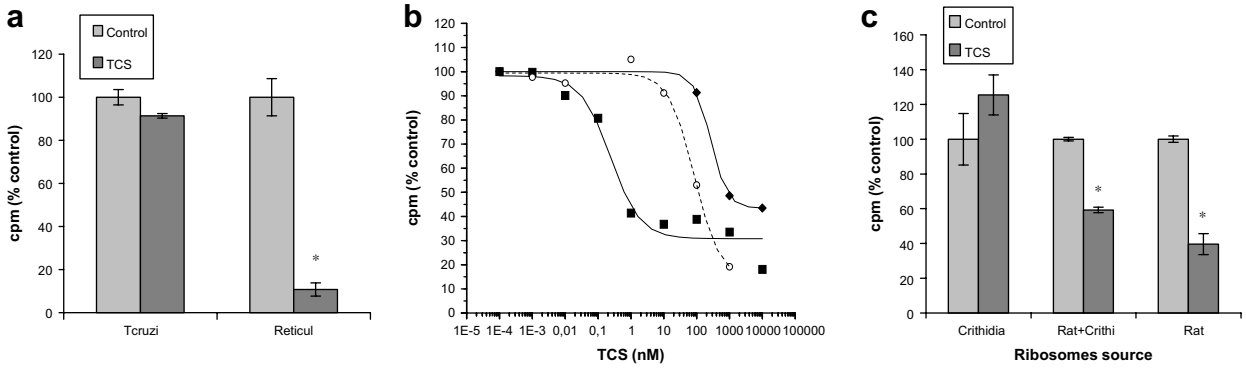

Fig. 2. (a) Effect of TCS on the *in vitro* polypeptide elongation activity by *T. cruzi* ribosomes (TCS 100 nM) and rabbit reticulocyte lysate (TCS 10 nM). Data are presented as media ± SD and asterisks indicate significant differences with control ($p < 0.05$). (b) Dose–response of the inhibitory effect of TCS on rat (black squares), *C. fasciculata* (white circles) and *T. cruzi* (black diamonds) ribosomes. (c) *In vitro* protein synthesis by *C. fasciculata* and/or rat ribosomes in the presence or absence of TCS 10 nM. Data are presented as media ± SD and asterisks indicate significant differences with control ($p < 0.05$).

Table 1
IC_{50} values of wt and mutated TCS on *in vitro* protein synthesis by rat, *C. fasciculata* and *T. cruzi* ribosomes

	IC_{50} (nM)		
	Rat	*C. fasciculata*	*T. cruzi*
wtTCS	0.21	89.24	236.82
MutTCS	1.35	946.02	1490.4
Ratio MutTCS/wtTCS	6.5	10.6	6.3

tein synthesis by ribosomes from the three different sources; rat, *T. cruzi* and *C. fasciculata*. Different amounts of TCS were used to obtain inhibition curves (Fig. 2b) and estimate the IC_{50} values for *in vitro* protein synthesis by rat, *C. fasciculata* and *T. cruzi* ribosomes (Table 1).

In order to rule out the presence of unknown TCS-inactivating activities in the trypanosomatid ribosome preparation, we performed assays with mixtures of rat and *C. fasciculata* ribosomes in the same reaction tube in the presence of 10 nM of TCS (a concentration which significantly inhibits rat ribosomes but has no effect on the *C. fasciculata* particles). As expected, we observed an intermediate reduction in the incorporated radioactivity (Fig. 2c), consistent with TCS acting on rat ribosomes but not on the *C. fasciculata* particles.

3.3. Trypanosomatid ribosomes are less affected by K173A/R174A/K177A TCS

Triple alanine subtitution at K173, R174 and K177 on TCS produces a decrease in the inhibitory effect of the toxin on reticulocyte lysate. This lower activity of the triple alanine mutated TCS (MutTCS) is consistent with a lower affinity to ribosomes, caused by an impairment of the interaction with the C-terminal end of P proteins (Chan et al., 2007). As it is shown in Fig. 3a the *T. cruzi* P proteins C-terminal peptides are slightly different from the mammalian ones, especially in the case of P0 (Levin et al., 1993). However, the DDD motif which has been involved in the interaction with TCS (Chan et al., 2007) is conserved. To test whether the resistance to TCS of trypanosomatid ribosomes is due to the inability of TCS to interact with the non-canonical C-terminal end of

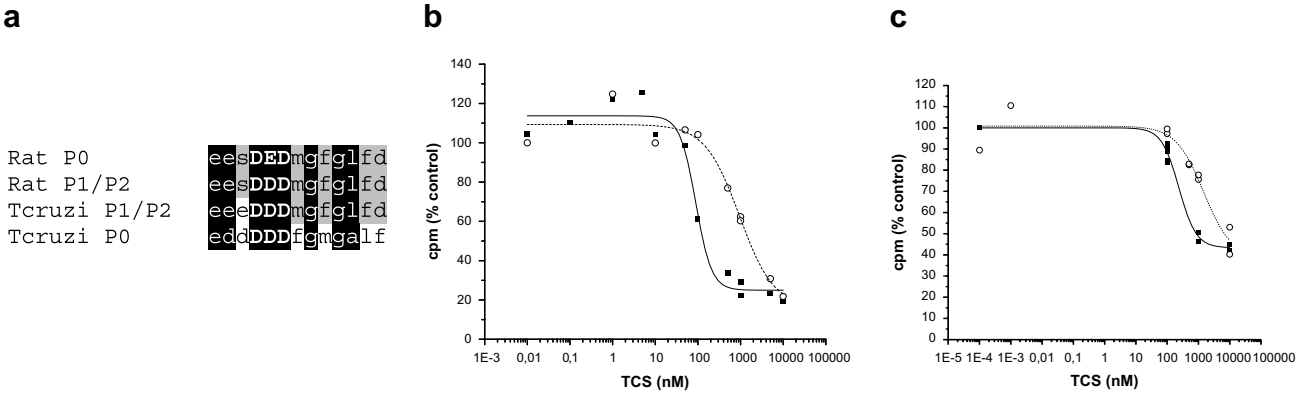

Fig. 3. (a) Sequence alignment of the 13 amino acids C-terminal peptide of ribosomal P proteins from rat and *T. cruzi*. Identical or conservative changes in all proteins are shown in black background. Residues partially conserved are shown in grey background. The DDD motif interacting with K173, R174 and K177 of TCS is shown in capital letters. (b and c) Effect of different concentrations of wtTCS (black points) and MutTCS (white points) on the protein synthesis by *C. fasciculata* (b) and *T. cruzi* (c) ribosomes, respectively. The corresponding IC_{50} values are shown in Table 1.

trypanosomatid P proteins, we have measured the IC_{50} values for wt and MutTCS towards rat, *C. fasciculata* and *T. cruzi* ribosomes. We observed that IC_{50} value for MutTCS is approximately 10 times higher than for wtTCS when assayed against *C. fasciculata* ribosomes (Fig. 3b and Table 1). A similar effect was observed with *T. cruzi* (Fig. 3c and Table 1), as well as rat ribosomes (Table 1). That the triple alanine mutation of TCS showed similar relative effects compared to wild-type TCS towards rat, *T. cruzi* and *C. fasciculata* ribosomes (Table 1) suggests that TCS binds similarly to rat and trypanosomatid P proteins. Our data support the conclusion that the resistance of trypanosomatid ribosomes to TCS is due to other ribosomal features rather than to the C-terminal end of P proteins. It is probable that multiple factors are acting sinergically to make the trypanosomatid ribosomes largely resistant to TCS, and probably also to other RIPs. Studies are currently underway to clarify these factors.

The action of TCS and other RIPs is to depurinate an invariant A residue (A^{4324} in rat) located in the so-called sarcin/ricin loop (SRL), a highly conserved region on the 28S rRNA of the large subunit. Even when the SRL is conserved in *T. cruzi* (Access code for 28S rRNA: Tc00.1047053422723.20 at www.genedb.org) and *C. fasciculata* (GenBank Access for 28S rRNA: Y00055), some interesting facts should be mentioned. First, the SRL in trypanosomatids form part of an 180b RNA fragment (28Sdelta fragment), due to the unusual processing of the 28S rRNA in these organisms (White et al., 1986). The absence of covalent bond between the rRNA containing SRL and the rest of the rRNA could have some effect on the relative location of the SRL in the context of the ribosome and/or its accessibility by TCS. Second, although the predicted secondary structure of the trypanosomatid 28Sdelta fragment is virtually identical to its mammalian counterpart (Liang et al., 2005), the sequence identity is relatively low (around 54% of identity percent comparing to rat).

Because the TCS-binding site on the ribosome is overlapped to the elongation factors binding site, it is conceivable the existence of particular features in the interaction of these soluble proteins with the ribosome in trypanosomatids.

In summary, the present work shows that trypanosomatid ribosomes are largely resistant to the action of TCS, suggesting the presence of important structural and functional differences between trypanosomatid and mammalian ribosomes in the region around the SRL. These differences could be exploited in the future for developing anti-parasitic drugs. In addition, the study of the toxicity of RIPs toward early diverging eukaryotes such as *T. cruzi* could be useful for understanding the detailed action mechanism of these toxins.

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