

Lactose derivatives are inhibitors of *Trypanosoma cruzi* trans-sialidase activity toward conventional substrates *in vitro* and *in vivo*

Rosalía Agustí², Gastón París³, Laura Ratier³,
Alberto C.C. Frasch³, and Rosa M. de Lederkremer^{1,2}

²CIHIDECAR, Departamento de Química Orgánica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria, Pabellón II, 1428 Buenos Aires, Argentina, and ³Instituto de Investigaciones Biotecnológicas-Instituto Tecnológico de Chascomús, Universidad Nacional de General San Martín y Consejo Nacional de Investigaciones Científicas y Técnicas, INTI, Av. General Paz s/n, Edificio 24, Casilla de correo 30, 1650 General San Martín, Argentina

Received on February 6, 2004; revised on March 15, 2004;
accepted on March 15, 2004

Chagas' disease, caused by *Trypanosoma cruzi*, affects about 18 million people in Latin America, and no effective treatment is available to date. To acquire sialic acid from the host glycoconjugates, *T. cruzi* expresses an unusual surface sialidase with trans-sialidase activity (TcTS) that transfers the sugar to parasite mucins. Surface sialic acid was shown to have relevant functions in protection of the parasite against the lysis by complement and in mammalian host cell invasion. The recently determined 3D structure of TcTS allowed a detailed analysis of its catalytic site and showed the presence of a lactose-binding site where the β -linked galactose accepting the sialic acid is placed. In this article, the acceptor substrate specificity of lactose derivatives was studied by high pH anion-exchange chromatography with pulse amperometric detection. The lactose open chain derivatives lactitol and lactobionic acid, as well as other derivatives, were found to be good acceptors of sialic acid. Lactitol, which was the best of the ones tested, effectively inhibited the transfer of sialic acid to *N*-acetylglucosamine. Furthermore, lactitol inhibited parasite mucins re-sialylation when incubated with live trypanosomes and TcTS. Lactitol also diminished the *T. cruzi* infection in cultured Vero cells by 20–27%. These results indicate that compounds directed to the lactose binding site might be good inhibitors of TcTS.

Key words: alternative substrates/HPAEC/inhibitors/
trans-sialidase/*Trypanosoma cruzi*

Introduction

To acquire sialic acid from the host glycoconjugates, *Trypanosoma cruzi* expresses a glycosylphosphatidylinositol-anchored trans-sialidase (TcTS) on its surface (Agustí

et al., 1998, 1997; Parodi *et al.*, 1992; Schenkman *et al.*, 1994). TcTS is a modified sialidase that preferentially catalyzes the transfer of α -2,3 linked sialic acid from host glycoconjugates to terminal β -galactosyl units of parasite mucins (Frasch, 2000; Schenkman *et al.*, 1991, 1993). The sialic acid on *T. cruzi* surface has relevant functions in protection of the parasite against the complement (Tomlinson *et al.*, 1994) and host cell invasion (Lopez *et al.*, 2002; Schenkman *et al.*, 1991). It has been shown recently that TcTS is a virulence factor of *T. cruzi* that induces transient thymic aplasia (Belen Carrillo *et al.*, 2000; Mucci *et al.*, 2002). In fact, DNA vaccination with TcTS sequences protects against the infection (Fujimura *et al.*, 2001; Katae *et al.*, 2002; Wizek *et al.*, 1998). In the African trypanosome *T. brucei*, the agent of diseases known as sleeping sickness in humans and nagana in cattle, trans-sialidase is expressed in the procyclic stage and sialylates the glycosylphosphatidylinositol anchor of procyclins, which cover all the parasite surface (Montagna *et al.*, 2002; Pontes de Carvalho *et al.*, 1993). However, the biological relevance of this process remains to be studied.

Given the essential roles of TcTS in infection and pathogenesis, this enzyme is a good target for the development of alternative chemotherapy against the parasite. Nevertheless, no specific inhibitor of TcTS is currently available. A sialidase inhibitor, analog to the oxocarbenium transition state of the reaction, 2,3-didehydro-2-deoxy-*N*-acetylneuraminic acid (Neu2en5Ac), has to be used ~100 times more concentrated to inhibit TcTS when compared to bacterial and viral sialidases. The 3D structure of TcTS was recently determined and the catalytic mechanism elucidated (Amaya *et al.*, 2003; Buschiazzi *et al.*, 2000, 2002). TcTS has two (sub)sites in the active center: the sialic acid-binding site and the galactose-binding site. The sialic acid (sub)site is present in all sialidases but in TcTS is somewhat different. The sugar enters in a tilted position compared with other sialidases, partially explaining the lack of inhibition observed with Neu2en5Ac. On the other hand, the lactose (sub)site is absent in all known sialidases and is formed on binding of substrate. Binding of the sialic acid moiety of the donor substrate in the TcTS reactive center triggers a conformational switch that creates the sugar acceptor-binding site for a terminal β -galactose. In the free enzyme, the Tyr119 points toward the floor of the catalytic pocket filling the space of sialic acid. On binding of the donor substrate, the Tyr119 side chain suffers a displacement leaving the sialic acid binding cleft and forming a stacking interaction with lactose, which is placed against the Trp312. In addition to creating the acceptor-binding site, a second consequence of the binding of sialic acid is a conformational switch of a Tyr residue at

¹To whom correspondence should be addressed; e-mail:
lederk@qo.fcen.uba.ar

position 342, which is the catalytic nucleophile making a covalent bond with the sialic acid intermediary state of the reaction (Watts *et al.*, 2003).

So far, the best donor and acceptor molecules known are sialic acid $\alpha(2,3)$ -linked to a β -galactose, and a terminal β -galactose respectively (Scudder *et al.*, 1993; Vandekerckhove *et al.*, 1992). *In vitro*, 3'-sialyllactose and lactose are widely used to assay for TcTS activity. *In vivo*, 3'-sialyl *N*-acetyllactosamine structures are present in a number of mammalian glycoconjugates while terminal β -galactosyl units are constituents of parasite mucins (Acosta-Serrano *et al.*, 2001). In the present study we tested lactose derivatives modified in the glucose constituent with the aim of understanding the contribution of the sugar linked to galactose in the trans-sialidase reaction. It was previously described (Buschiazzo *et al.*, 2002) that the 3-OH of the reducing glucose in lactose is involved in water-mediated hydrogen bonds with the enzyme. We found that lactitol is an excellent acceptor of sialic acid but sialyllactitol was unable to act as a donor substrate. Lactitol was able to inhibit the trans-sialidase reaction toward conventional substrates *in vitro* and to interfere with parasite infection in cultured cells. These results suggest that the lactose-binding site of TcTS might be used for the design of inhibitors to be tested against trypanosomal infections.

Results

Testing of lactose derivatives as sialic acid acceptors in the trans-sialidase reaction

Structures for the compounds analyzed as acceptor substrates for TcTS are shown in Figure 1. Optimal conditions for sialylation were obtained by following the reaction by high pH anion-exchange chromatography (HPAEC) using 1 mM 3'-sialyllactose as donor and 1 mM each of the substrate acceptors tested. In all cases, the reaction was fast and reached the equilibrium in about 15 min. Lactitol is an acceptor of sialic acid, showing 80% transference of sialic acid. This value was calculated from the amount of sialyllactitol formed with respect to the sum of sialyllactitol and sialyllactose remaining after the reaction. The sialyllactitol (t_R 3.8 min) formed was clearly separated from sialyllactose (t_R 9.5 min) and free sialic acid (t_R 5.6 min), which was not detected (Figure 2A). Under the conditions used for HPAEC (condition 1, see *Materials and methods*), the lactose formed in the reaction and the lactitol that remained not sialylated were eluted before 2 min. Lactobionic acid also resulted a good sialic acid acceptor (65% transference). However, different conditions were used (condition 2) because the double negatively charged sialyllactobionic acid requires an increase in the elution force of the solvent system (Figure 2B).

When lactobionic acid was incubated with TcTS and 3'-sialyllactose, a peak eluted at 10.50 min. To verify the identity of this peak, fractions eluted at this time were collected, decationized, subjected to mild acid hydrolysis under conditions that hydrolyze the sialic acid only, and rechromatographed under the same conditions. The peak at 10.50 min disappeared, and two peaks corresponding to sialic acid and lactobionic acid were detected (data not

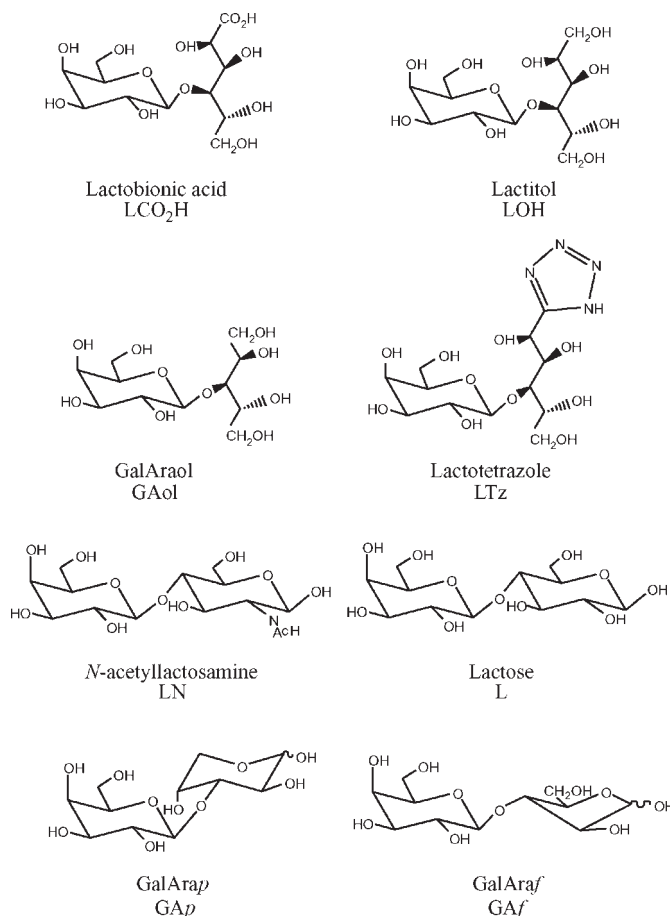


Fig. 1. Structure of the compounds analyzed as substrates for the TcTS. Lactobionic acid (LCO₂H), 4-*O*- β -D-galactopyranosyl-D-gluconic acid; Lactitol (LOH), 4-*O*- β -D-galactopyranosyl-D-glucitol; GalAraol (GAol), 3-*O*- β -D-galactopyranosyl-D-arabinitol; lactotetrazole (LTz), 5-[3'-*O*- β -D-galactopyranosyl-D-gluco-pentitol-1'-yl]-tetrazole; *N*-acetyllactosamine (LN), 2-acetamido-2-deoxy-4-*O*- β -D-galactopyranosyl-D-glucopyranose; lactose (L), 4-*O*- β -D-galactopyranosyl-D-glucose; GalArap (GAp), 3-*O*- β -D-galactopyranosyl-D-arabinopyranose; GalAraf (GAf'), 3-*O*- β -D-galactopyranosyl-D-arabinofuranose.

shown). The sialylation of lactobionic acid and lactitol showed that glucose is not an indispensable component of the acceptor molecule because both derivatives of lactose, the corresponding alditol and the C-1 oxidized aldonic acid, proved to be substrates in the trans-sialidase reaction. Sialylation of *N*-acetyllactosamine was used as the positive control of the reaction (Figure 2C). Controls without the enzyme were analyzed for each substrate.

The next question was if the chain length of the sugar linked to the β -galactose substrate is important for TcTS activity. 3-*O*- β -D-Galactopyranosyl-D-arabinose (GalAra, Figure 1) was tested as acceptor. When the substrate GalAra was analyzed by HPAEC using condition 5, the 3-*O*- β -D-galactopyranosyl-D-arabinopyranoses (GalArap, t_R 14.10) and the 3-*O*- β -D-galactopyranosyl-D-arabinofuranoses (GalAraf, t_R 40.40) were detected (t_R of lactose 22.30, data not shown). The furanose structure for the reducing end was assigned for the disaccharides with the

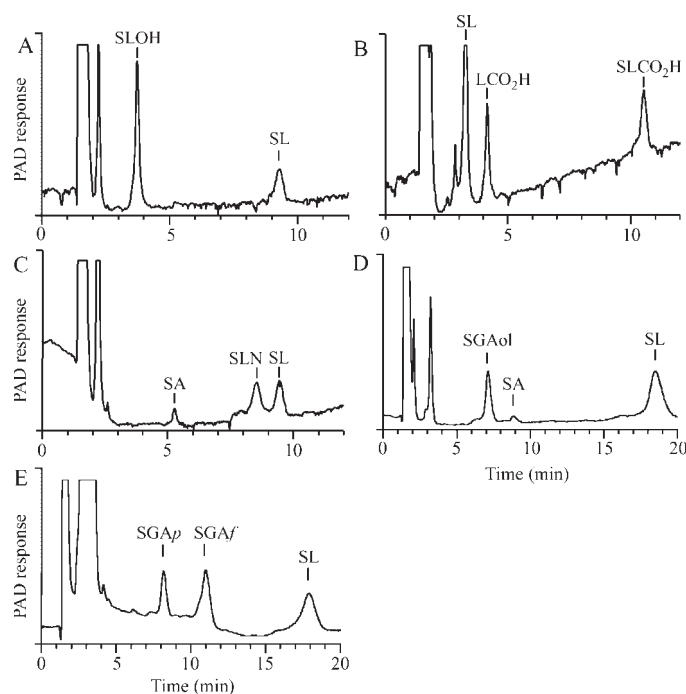


Fig. 2. Analysis of substrates for the trans-sialidase from *Trypanosoma cruzi*. Compounds shown in Figure 1 at 1 mM concentrations were incubated with 1 mM 3'-sialyllactose and trans-sialidase for 30 min and the reaction mixtures analyzed by HPAEC-PAD as described under *Materials and methods*. (A) Substrate, lactitol, a PA-100 column under condition 1 was used. (B) Substrate, lactobionic acid; a PA-100 column under condition 2 was used. (C) Substrate, *N*-acetyllactosamine, conditions as for A. (D) Substrate GAol; conditions as for A but with a new column. (E) Substrate, GA, a PA-10 column under condition 3 was used. SA, sialic acid; SLOH sialyllactitol; SLCO₂H, sialyllactobionic acid; SL, sialyllactose; SLN, sialyl *N*-acetyllactosamine; SGAol, sialyl GalAraol; SGA_p, sialyl GalAra_p; SGA_f, sialyl GalAra_f.

highest retention time because we have previously shown that isomeric furanose disaccharides are retained for longer times on HPAEC (Salto *et al.*, 2000). On the other hand, the ratio of pyranoses to furanoses is in agreement with the integration for the anomeric centers in the nuclear magnetic resonance (NMR) spectra (Othegui *et al.*, 1990). HPAEC conditions were optimized using a CarboPac PA-10 column that provides better resolution for compounds with the same molecular weight.

When GalAra was incubated with 3'-sialyllactose and trans-sialidase, two sialylated products were observed corresponding to the saccharides with the arabinopyranose and the arabinofuranose at the reducing end respectively (42% total transference, Figure 2E). Considering that the presence of an arabinose residue instead of glucose did not prevent trans-sialylation, it was interesting to investigate if the same happened when arabinitol was present instead of glucitol, the constituent of lactitol. 3-*O*-β-D-galactopyranosyl-D-arabinitol (GalAraol, Figure 1) was prepared by reduction of GalAra with NaBH₄. The corresponding alditol was characterized by ¹³C-NMR that showed only one anomeric carbon at 103.8 ppm and three primary carbons at 63.01 ppm, 61.82 ppm, and 60.41 ppm. Figure 2D shows that GalAraol is also a good acceptor in the trans-sialidase

reaction (74% transference). In fact, the alditol was a better acceptor than the disaccharide GalAra. These results indicate that the length of the carbon chain of the residue to which the galactose is β-linked is not restricted to six carbon atoms for the reaction to take place.

Another lactose derivative with its C-1 modified with a tetrazole residue, was also tested as a substrate for TcTS. Lactotetrazole (Figure 1) was obtained by treatment of the perbenzoylated compound (Othegui *et al.*, 1990) with sodium methoxide in methanol. Although tetrazole is a weak acid similar in strength to acetic acid (pK_a = 4.76), under the conditions used, lactotetrazole was eluted in the region of the neutral lactose derivatives (*t*_R 2.60 min, lactose *t*_R 3.20 min, condition 4). Presence of a sialylated lactotetrazole was not detected (data not shown).

Different extent of sialic acid transfer was observed with the acceptor substrates used. Therefore, we estimated the apparent *K*_m for each acceptor to quantify the relative affinity of TcTS for the substrates (Figure 3). The rate of the enzymatic reaction with respect to the different substrates was expressed as the percentage of sialylated substrate synthesized over a period of 15 min. Incubations were carried out with 1 mM 3'-sialyllactose, 40 ng trans-sialidase and different substrate concentrations. As can be observed in Figure 3, lactitol has the lowest *K*_m value (0.26 mM). The value obtained for *N*-acetyllactosamine was in the range of that for lactose (Ribeirao *et al.*, 1997). Lactobionic acid and the alditol GalAraol seem to be poorer acceptors having *K*_m values four to six times larger than that of lactitol. The *K*_m of the disaccharide GalAra was not determined because the presence of the two configurations in the arabinose residue did not allow an accurate measurement.

Sialyllactose derivatives as donors in the trans-sialidase reaction

For most of the known acceptors, trans-sialidase is a fully reversible enzymatic reaction. Once the sialylated product is synthesized, it could also act as sialic acid source in the reverse reaction, whereas the desialylated lactose behaves as acceptor. To determine if sialylated derivatives of lactitol, lactobionic acid, and *N*-acetyllactosamine could also be sialic acid donors in trans-sialidase reaction, the corresponding fractions of each chromatogram in Figures 2A, B, and C were collected, desalted, and incubated with the enzyme in the presence of lactose.

Sialyllactobionic acid was also prepared by using fetuin as the donor of sialic acid as described under *Materials and methods*. When sialylated lactobionic acid was used as donor, it was completely consumed, deriving in the formation of sialyllactose and lactobionic acid (Figure 4B). In contrast, when sialyllactitol was used as donor, sialylation of lactose was not observed at any of the concentrations of sialyllactitol used, although the presence of the donor could clearly be observed in the chromatograms (Figure 4A). An intermediate situation was found when sialyllactosamine was used as the donor. Figure 4C shows the presence of both compounds, sialylated lactose, and the remaining sialyllactosamine. These results correlated with the app*K*_m values obtained. The worse a compound behaved as an acceptor in the trans-sialidase reaction, the better its sialylated

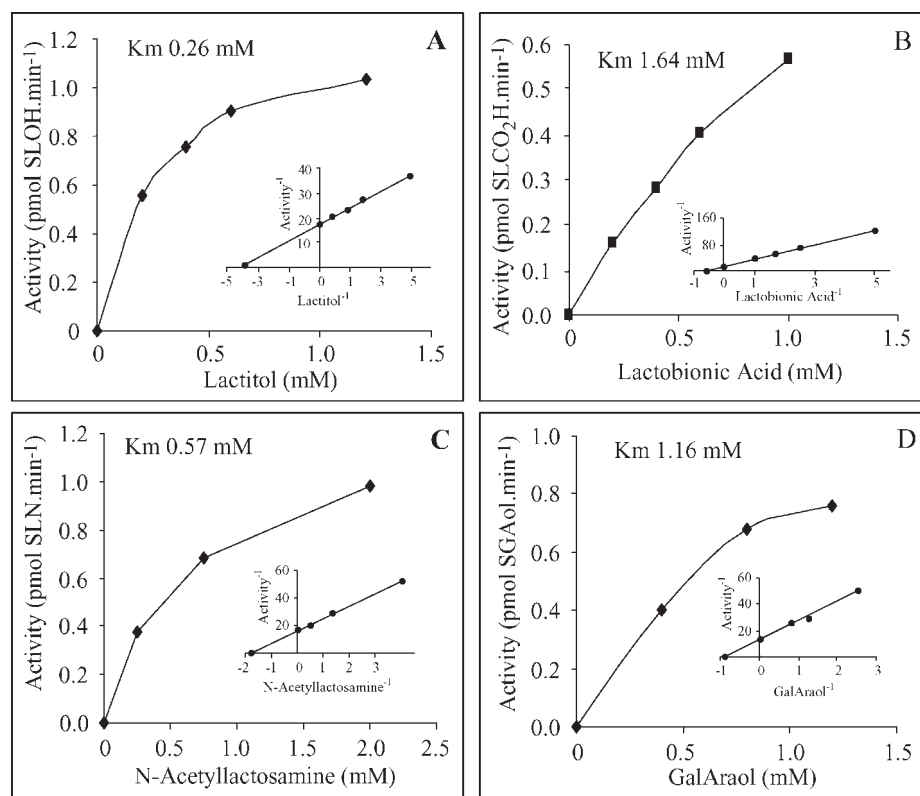


Fig. 3. Michaelis-Menten constants of different substrates. Different concentrations of acceptor substrates were incubated with trans-sialidase and 1 mM 3'-sialyllactose and analyzed by HPAEC-PAD under the conditions specified in Figure 2. (A) Lactitol; (B) lactobionic acid; (C) *N*-acetylglucosamine; and (D) GalAraol. Apparent K_m values were calculated from Lineweaver-Burk plots show in the insets.

derivative behaved as a donor in the reverse reaction. In the case of sialyllactitol, the absence of transference when lactose is used as an acceptor suggests that lactitol is preferably sialylated instead of lactose and that once the sialyllactitol is formed, the reaction cannot be forced backward.

Inhibition of sialylation of N-acetylglucosamine by lactose derivatives

To find out whether the lactose derivatives used in this study were capable of inhibiting sialylation of *N*-acetylglucosamine, different concentrations of derivatives were tested in transfer reactions containing 1 mM 3'-sialyllactose as a donor, 1 mM *N*-acetylglucosamine as an acceptor, and TcTS (Table I, Figure 5A). When equal concentrations of lactitol (1 mM) and *N*-acetylglucosamine were used as acceptors of sialic acid, lactitol is sialylated preferentially (Table I), in accordance with the calculated values of $appK_m$. Total sialic acid transfer (Table I) refers to the sialyllactose consumed after the reaction. Sialyllactitol accounted for 74.3% of the amount transferred (Table I).

When lactobionic acid was tested as an inhibitor of the reaction, equal concentrations of *N*-acetylglucosamine and lactobionic acid resulted in a reduction of sialylation of *N*-acetylglucosamine caused by the sialylation of lactobionic acid (Table I, Figure 5B). However, both lactobionic acid and *N*-acetylglucosamine were sialylated to the same extent

(Table I). Under the conditions used, the sialyllactobionic acid was strongly retained by the column and was eluted as a broad peak after 25 min. Total transfer of sialic acid from 3'-sialyllactose was inhibited by about 40%. To study the ability of lactobionic acid to function as inhibitor of the sialylation of lactose, fetuin was used as the donor of sialic acid. Glucuronic acid was used as the internal standard in the HPAEC (Figure 6A). A 43% decrease in the formation of 3'-sialyllactose was observed while the peak corresponding to sialyllactobionic acid was detected (Figure 6B).

GalAraol and GalAra were also inhibitors for the sialylation of *N*-acetylglucosamine (Figure 5C and 5D). It is interesting to notice that even though formation of sialyllactosamine was effectively inhibited, the total transfer of sialic acid remained constant in the case of GalAra and even increased in the case of GalAraol (Table I). When lactotetrazole was tested as an inhibitor, a decrease in the sialylation of *N*-acetylglucosamine occurred, even though it does not compete for the use of sialic acid (Figure 5E).

Lactitol inhibited parasite sialylation in vivo

The *in vivo* effect of lactitol on mucin resialylation by TcTS was analyzed measuring the total sialic acid content of parasites. Bovine serum albumin (BSA)-parasites were collected in serum-free medium with BSA. In this medium, parasite sialylation is prevented due to the absence

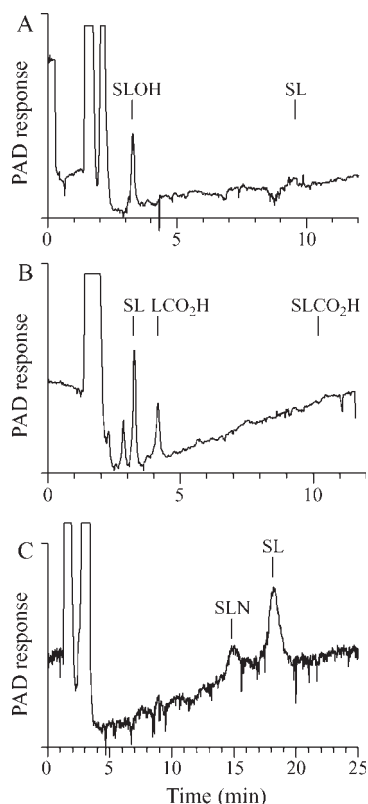


Fig. 4. Analysis by HPAEC-PAD of donors for the trans-sialidase reaction. Sialylated derivatives of lactitol (A), lactobionic acid (B), and *N*-acetylglucosamine (C), obtained by sialylation of the acceptor substrates (see Figure 2) were incubated with lactose and trans-sialidase, and the reaction mixtures analyzed by HPAEC. A CarboPac PA-100 column was used for chromatograms 4A and 4B using conditions 1 and 2 (see *Materials and methods*), respectively. A CarboPac PA-10 column was used for chromatogram 4C under condition 4. SLOH, sialyllactitol; SL, sialyllactose; LCO₂H, lactobionic acid; SLCO₂H, sialyllactobionic acid; SLN, sialyllactosamine.

of sialic acid donors. Total sialic acid content was determined by the thiobarbituric acid method with high-performance liquid chromatography (HPLC) analysis in BSA-parasites and in parasites that were resialylated with recombinant TcTS and sialyllactose as donor. Both conditions were performed in the absence or presence of lactitol (see *Materials and methods* for further details). Three independent experiments for each of the conditions were done (Table II).

BSA-parasites incubated with or without lactitol have about the same amount of sialic acid in all three experiments (from 5 to 9 pmol per million of parasites, Table II). Parasites collected in BSA and treated with TcTS and sialyllactose have two to five more times sialic acid as compared with parasites that were not treated with the enzyme. When trypanosomes were resialylated with TcTS in the presence of lactitol, values were in all cases lower than those of parasites treated with TcTS in the absence of the inhibitor (Table II). In two of the three experiments, lactitol completely prevented parasite sialylation by TcTS, maintaining the sialic acid levels similar to those of parasites collected in BSA (see values from experiments 1 and

3 in Table II). In the experiment 2 (Table II) parasite resialylation by trans-sialidase was more pronounced than in the other two experiments. This might be due to the complexity of the experimental procedure or to the accepted variation in these biological systems. Taken together, these experiments showed that lactitol prevents trypanosome resialylation by TcTS.

Effect of lactitol in the infection of culture mammalian cells by T. cruzi

Sialic acid was suggested to influence the infection of host cells by the parasite. Thus the identification of acceptor molecules that efficiently compete with *N*-acetylglucosamine for the sialic acid transferred by TcTS prompted us to test their effect in a culture cell infection assay. BSA-parasites (see previous discussion) were incubated with Vero cells; after 3 days, the number of infected cells were counted (see *Materials and methods* for details). On contact with Vero cells, parasites might incorporate sialic acid from the glycoconjugates present on the surface of the host cells. In this step lactitol might prevent parasite sialylation and thus prevent cell infection. Under a second condition, BSA-parasites were resialylated with TcTS and sialyllactose before used to infect Vero cells. Presence of lactitol was maintained through all the procedure.

Under the two conditions, BSA-parasites and BSA-parasites resialylated with TcTS, lactitol was able to inhibit infection to a similar extent (20–27% of inhibition, Table III). The experiments were repeated three to seven times (see Table III), and the differences observed between control and lactitol-treated groups were statistically significant ($p=0.001$). No morphological alterations in both, parasites and Vero cells, were observed throughout the experiments under any of the conditions used. As a control, parasites collected in BSA were incubated with lactose at the same concentration used for lactitol. No effect on the infection by *T. cruzi* was observed with lactose (Table III).

We also tested the effect of lactitol on parasites that were collected in fetal bovine serum (FBS) and then used directly to infect cells. These trypanosomes should be fully sialylated and therefore showed a higher percentage of infection than BSA-trypanosomes. When lactitol was added, a 32% decrease in the infection rate was observed (Table III). No morphological alterations in parasites or Vero cells was observed under any of the conditions used.

Discussion

In this article we show that there are acceptor molecules that efficiently compete with *N*-acetylglucosamine/lactose for the sialic acid, inhibiting TcTS activity toward conventional substrates *in vitro* and *in vivo*. Lactitol is the most efficient from the ones tested and was the only one that once sialylated was unable to act as a donor of sialic acid. Other lactose derivatives having different structures replacing the glucose were also alternative substrates for TcTS. Among them is lactobionic acid, which in a previous work was reported not to be an acceptor in the trans-sialidase reaction (Scudder *et al.*, 1993). However, in another study, using [¹⁴C]-lactose as an acceptor, it was reported that lactobionic

Table I. Inhibition of sialic acid transfer to *N*-acetylglucosamine by different acceptor substrates.

Acceptor substrate	Concentration (mM)	S-LN (%)	S-substrate ^a (with 1 mM LN) (%)	Total sialic acid transfer (%)	S-substrate (without LN) ^b (%)
Lactitol (LOH)	0	40.5 (100)	0	40.4 (100)	—
	0.5	26.4 (43.1)	34.9 (56.9)	61.3 (100)	62.4
	1.0	16.2 (25.7)	46.8 (74.3)	63.0 (100)	68.0
Lactobionic acid (LCO ₂ H)	0	36 (100)	0	36 (100)	—
	0.4	25 (83.3)	5 (16.7)	30 (100)	21
	1.0	13 (59.1)	9 (40.1)	22 (100)	42
Galactosyl-arabinose (GA)	0	48.2 (100)	0	48.2 (100)	—
	1.0	46.5 (100)	0	46.5 (100)	42 ^c
	6.0	11.8 (18.1)	53.5 (81.9)	65.3 (100)	ND
Galactosyl-arabinitol (GAol)	0	43.5 (100)	0	43.5 (100)	—
	0.5	30.4 (52.6)	27.4 (47.4)	57.8 (100)	30.4
	1.0	27.5 (42.4)	37.4 (57.6)	64.9 (100)	52
Lactotetrazole (LTz)	0	47.5 (100)	0	47.5 (100)	—
	0.8	35.9 (100)	0	35.9 (100)	0
	3.4	23.1 (100)	0	23.1 (100)	0

Different concentrations of substrates were incubated with 1 mM 3'-sialyllactose (donor-substrate), 1 mM *N*-acetylglucosamine, and trans-sialidase. The reaction products were analyzed by HPAEC. Percentages were calculated from the amount of sialylated substrate with respect to the total amount of sialylated compounds after the reaction. In parentheses the percentage of sialic acid transfer to acceptor-substrate relative to total sialic acid transfer. ND, not determined.

^aSialylated-substrate.

^bIncubations without *N*-acetylglucosamine were performed for comparison and analyzed by HPAEC-PAD under conditions of Figure 2.

^cPercentages of both forms, furanose and pyranose.

acid inhibited the formation of sialyl [¹⁴C]-lactose in a concentration-dependent manner (Vandekerckhove *et al.*, 1992), thus suggesting that it was an acceptor in the trans-sialidase reaction. In this respect, we elucidated the controversy regarding the substrate properties of lactobionic acid by unquestionably showing its sialylation and donor properties of the sialylated lactobionic acid. From the biochemical point of view, it is interesting to note that the glucose ring in lactose was not required at all for the transfer reaction to take place. Thus these results confirm that what is recognized in the donor/acceptor molecule, are the terminal galactose and the β-linkage (Paris *et al.*, 2001).

Previous crystallographic studies showed that the OH-3 of the glucose in lactose is involved in water-mediated hydrogen bonding with Arg311 (Buschiazzo *et al.*, 2002). We found that the reducing sugar might be also replaced by an alditol. More important, lactitol is the preferred acceptor in the presence of lactose/*N*-acetylglucosamine, further supporting the idea that the open chain in fact favors the transfer reaction. Similar results have been obtained with *T. congolense* trans-sialidase using lactitol as acceptor in a competitive assay with 2'-(4-methylumbelliferyl) galactoside (MUGal) (Tiralongo *et al.*, 2003). Thus the alditol would be more flexible for adopting a conformation that favors hydrogen bond interactions with the enzyme. Also the alditol GalAraol was more efficient than GalAra for preventing sialylation of *N*-acetylglucosamine. The fact that no free sialic acid was observed with lactitol as acceptor

indicates that the sialic acid is efficiently transferred, instead of being hydrolyzed. So far, free sialic acid was always observed with all other acceptor molecules tested, indicating that although TcTS is a very efficient transferase, some hydrolytic activity was detectable. This was not the case with lactitol as acceptor, or at least not at the level of detection used. When sialyllactitol was used as donor, no transfer to *N*-acetylglucosamine could be detected (Figure 4A). A probable explanation of this result is that the sialic acid is rapidly transferred backward to lactitol from the acceptor substrate because TcTS has a higher affinity for lactitol than for *N*-acetylglucosamine.

TcTS 3D structure revealed the existence of two (sub)-sites within the catalytic region of the enzyme (Buschiazzo *et al.*, 2002). One is involved in binding sialic acid present in the donor molecule. This site proved to be somewhat different to that of other sialidases. Some of the amino acid residues present in TcTS make the sialic acid ring enter in a tilted position as compared with TrSA. Whether these, and other structural differences (see Buschiazzo *et al.*, 2002) are enough for the design of specific TcTS inhibitors directed to the sialic acid-binding site, remains to be studied. A 2,3 difluor derivative of sialic acid used to identify a covalent sialyl-enzyme intermediate was found to inactivate wild-type TcTS, although high concentrations (20 mM) were necessary for complete inactivation (Watts *et al.*, 2003). In the present work, the second (sub)site of the enzyme was analyzed as a possible target of TcTS inhibitors. This site binds the terminal galactose accepting the transferred

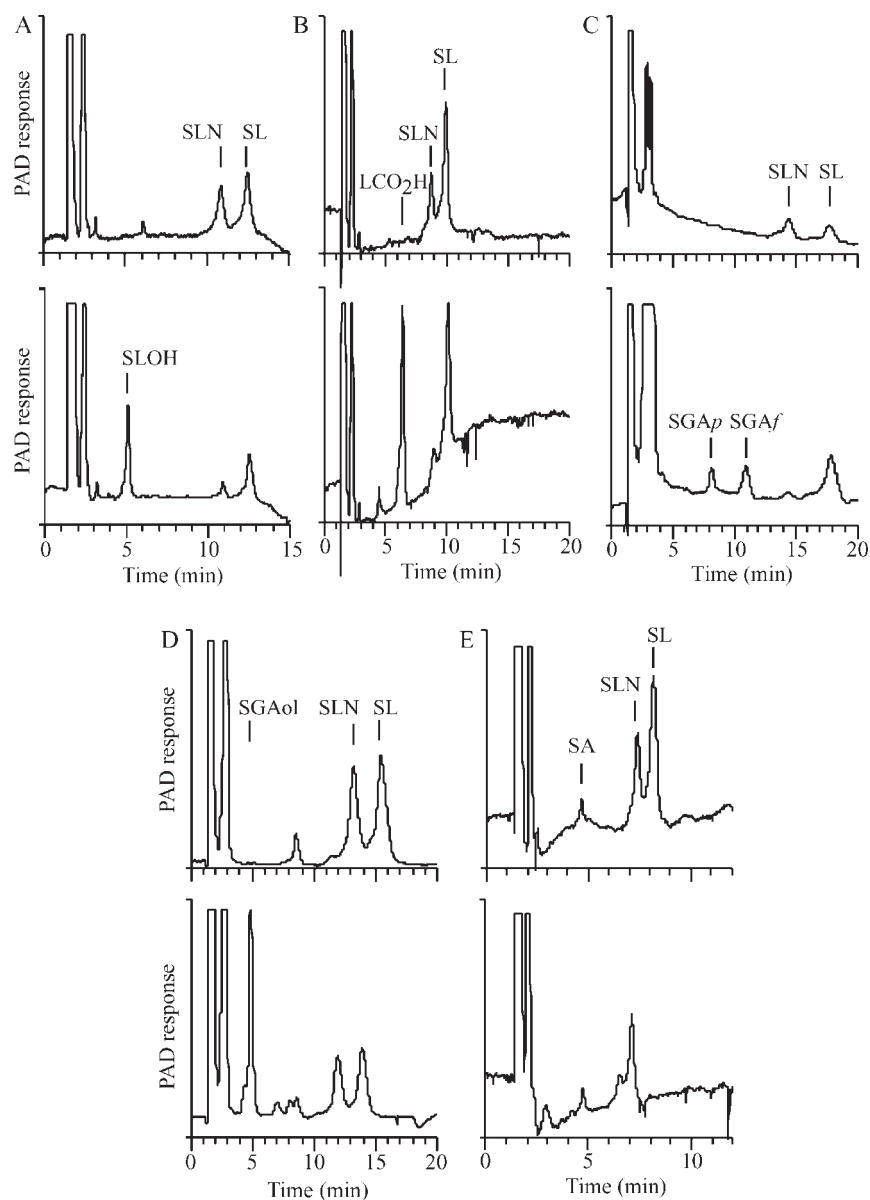


Fig. 5. Inhibition of sialylation of *N*-acetylglucosamine by different acceptor substrates. Reaction mixtures in 20 mM Tris (pH 7) buffer containing 1 mM 3'-sialyllactose (SL), 1 mM *N*-acetylglucosamine (LN), and 40 ng trans-sialidase were incubated with 1 mM of the competitive inhibitor for 30 min at 25°C and analyzed by HPAEC-PAD. For all panels, the upper chromatogram represents the *N*-acetylglucosamine sialylation (SLN) in the absence of inhibitor. Compounds used were: lactitol (A), lactobionic acid (LCO₂H, B), 3-O-β-D-galactopyranosyl-D-arabinose (C), 3-O-β-D-galactopyranosyl-D-arabinitol (D), lactotetrazole (E). A CarboPac PA-100 column was used for chromatograms of A, B, and E under conditions 2, 2, and 1, respectively. A CarboPac PA-10 column was used for chromatograms of C and D under condition 4. SLOH, sialyllactitol; SGAoI, sialyl 3-O-β-D-galactopyranosyl-D-arabinitol; SGAp, Sialyl 3-O-β-D-galactopyranosyl-D-arabinopyranose; SGAf, sialyl 3-O-β-D-galactopyranosyl-D-arabinofuranose.

sialic acid and involves a groove between Trp312 and Tyr119 that is present in trans-sialidases but not in sialidases.

Lactitol is so far the first example of a compound other than antibodies (Leguizamon *et al.*, 1994; Pitcovsky *et al.*, 2002) able to inhibit TcTS reaction toward substrates like lactose/*N*-acetylglucosamine. Interestingly, the galactose target site is only formed on binding of the sialic acid donor. Thus we assume that once the donor of sialic acid is bound to the active site and the site for the galactose is formed, lactitol is able to successfully compete with other

acceptors in the medium. In fact, it is able to compete with lactose/*N*-acetylglucosamine, which are widely used as acceptors to test for biochemical TS activity *in vitro*. Interestingly, and as predicted from the *in vitro* inhibition experiments, lactitol is able to prevent parasite sialylation in culture medium (Table II). In accordance, lactitol has also an effect on the infection of mammalian cells (Table III), as expected from previous results (Schenkman *et al.*, 1993) indicating that sialic acid on the parasite surface is required for this purpose. Nevertheless, the decrease in cell infection, 20–27% compared to control, is far from complete. Because

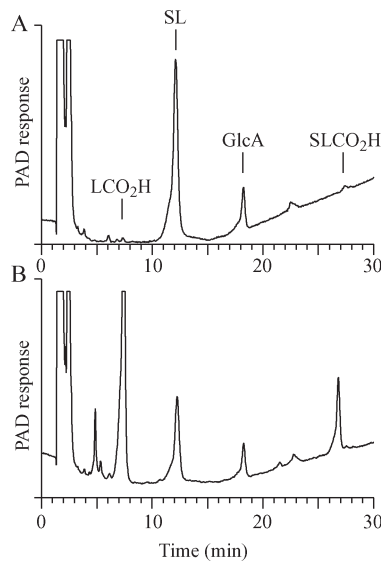


Fig. 6. Lactobionic acid as an inhibitor of the sialylation of lactose. A mixture containing 20 μ l lactose (10 mM), 20 μ l fetuin (10 mM in sialic acid), and 60 ng trans-sialidase was incubated with (B) or without (A) 20 μ l lactobionic acid (10 mM) in a total volume of 200 μ l buffer Tris (pH 7), at room temperature for 1 h. After filtration through a Sephadex G-25 P-10 column, the included volume was analyzed by HPAEC-PAD using glucuronic acid as an internal standard. The chromatograms were performed in a CarboPac PA-100 column under condition 1. SL, sialyllactose; LCO₂H, lactobionic acid; SLCO₂H, sialyllactobionic acid; glcA, glucuronic acid.

parasite desialylation is not complete, the sialic acid remaining on its surface might be enough for parasites to invade the host cell. This is also in accordance with previous results showing that partially desialylated parasites were still able to invade mammalian cells (Schenkman *et al.*, 1991, 1993). It is relevant to point out that a large number of parasites has to be used in the invasion experiments (200:1 trypanosomes:cultured mammalian cells) to obtain a number of infected cells amenable for quantification. These conditions are not comparable to those in nature, in which a few parasites deposited on the skin with the feces of the insect vector are enough to infect a human being.

Presently the best alternative to control Chagas' disease is to prevent the infection. This is done by controlling the triatomines and by testing for *T. cruzi* presence in blood used for transfusions. Once humans are infected, there are two products that are effective (albeit toxic) during the acute phase of the infection: nifurtimox and benznidazole (Cerecetto and Gonzalez, 2002). The effectiveness of these chemicals during the chronic phase of the infection is still under discussion (Kirchhoff, 2003; Urbina and Docampo, 2003). Thus finding new targets for chemotherapy of *T. cruzi* infection is needed. Trans-sialidase, a virulence factor that induces apoptosis in cells from the immune system (Leguizamon *et al.*, 1999; Mucci *et al.*, 2002), is a good alternative for the design of inhibitors. In this context, lactitol is a very innocuous molecule with an LD₅₀ of about 10 g/kg weight in mice (Okazaki *et al.*, 1994). However, lactitol is a small molecule, being quickly eliminated from blood. Our results provoke further investigations on the

Table II. Total amount of sialic acid in parasites in the presence or absence of lactitol

	BSA	BSA-Lactitol	BSA-TS	BSA-TS-Lactitol
Experiment 1	7.6	8.3	23.9	7.1
Experiment 2	8.9	6.4	33.3	26.7
Experiment 3	6.5	4.8	14.7	6.2

Total sialic acid was determined by the thiobarbituric acid method. BSA-trypomastigotes were produced as explained in *Materials and methods* and incubated with or without 3 μ g trans-sialidase (TS) plus 1 mM 3'-sialyllactose at 37°C for 30 min in the presence (Lactitol) or absence of 10 mM lactitol. Results are expressed as pmol of sialic acid per 10⁶ parasites.

Table III. Effect of lactitol on *T. cruzi* infection

	BSA	BSA-TcTS	FBS
Control	100.3 \pm 5.8 (7)	108.1 \pm 19.3 (5)	100.0 \pm 5.3 (3)
Lactitol	79.8 \pm 17.7 (7)	73.1 \pm 21.3 (5)	67.9 \pm 11.8 (3)
Lactose	95.7 \pm 8.2 (3)	ND	ND

Tissue culture trypanosomes grown in 0.2% BSA-MEM medium (BSA) were incubated at 37°C for 30 min before infection under the following conditions: untreated (control), 10 mM lactitol (Lactitol), and 10 mM lactose (Lactose). BSA parasites were incubated with 3 μ g recombinant trans-sialidase plus 1 mM sialyllactose (BSA-TcTS) with or without 10 mM lactitol. In a third group of experiments parasites were directly collected in FBS and used for cell invasion experiments. Parasites were washed and incubated overnight with Vero cells (2×10^6 parasites per well) with or without 10 mM lactitol or 10 mM lactose. The percentage of infected cells was quantified microscopically at 400 \times by staining with May-Grünwald-Giemsa in triplicate samples. Percentage of infection between BSA and BSA-lactitol was statistically significant ($p = 0.001$). Similar results were obtained for BSA-TcTS and BSA-TcTS-lactitol trypomastigotes ($p = 0.001$) and infections with parasites collected in FBS. The difference in infection between BSA-control and BSA-lactose was not statistically significant ($p = 0.2728$). Results are expressed as the mean \pm SE of the percentage of infected cells, normalized to the mean values obtained with control parasites, which were considered 100% of infection. Each experiment was done in triplicate. The number of independent experiments for each of the conditions tested is indicated in parentheses. ND, not determined.

design and testing of lactitol-based derivatives with a longer half-life in blood for testing against trypanosomal infections.

Materials and methods

Oligosaccharide substrates

Lactose was from Calbiochem (San Diego, CA). Lactitol, lactobionic acid, fetuin, Neu2en5Ac, N-acetyllactosamine, and 3'-sialyllactose were purchased from Sigma (St. Louis, MO). GalAra and GalAraol were prepared from 2,3,5,6,2',3',4',6'-octa-O-acetyllactobionitrile (D'Accorso *et al.*, 1988) and 5-[3'-O- β -D-galactopyranosyl-D-glucopentitol-1'-yl]-tetrazole (lactotetrazole, LTz) was prepared from 5-[1',2',4',5',2'',3'',4'',6''-octa-O-benzoyl-3'-O- β -D-galacto-

pyranosyl-D-gluco-pentitol-1'-yl]-tetrazole (Othegui *et al.*, 1990). The acylated substrates were a generous gift from Norma B. D'Accorso (University of Buenos Aires).

Trypanosomes

T. cruzi trypomastigotes, the form of the parasite that is liberated from infected mammalian cells, strain CL-Brener (Zingales *et al.*, 1997), were collected from supernatants of infected Vero cell cultures. Vero cells were grown in minimum essential medium (MEM, Sigma) with penicillin and streptomycin (Sigma) and supplemented with 3% FBS-Natocor at 37°C in 5% CO₂. Desialylated parasites were collected in BSA (Sigma) using a modified protocol based on Schenkman *et al.* (1993). Subconfluent cultures of Vero cells grown in 75-cm² flasks were infected with 15 × 10⁶ trypomastigotes and the cells were maintained in 3% FBS-MEM for three days after infection. Cells were washed twice with phosphate buffered saline (PBS) and MEM supplemented with 0.2% BSA, 20 mM HEPES, pH 7.4 (Sigma) (0.2% BSA-MEM). At day 5, supernatants were centrifuged 10 min at 4000 rpm (SS-34 Sorvall) and incubated at 37°C, 3 h to allow motile trypomastigotes to swim up from the pellet. Medium was collected, and parasites were used for cell invasion experiments.

Expression of recombinant TcTS in E. coli

A clone containing the TcTS gene (pTrcTS611/2; Buschiazzi *et al.*, 1997) was used to transform *Escherichia coli* BL21 (DE3) pLysS (Novagen, Madison, WI). After growth in terrific broth modified (Sigma) containing 100 µg/ml ampicillin (Sigma) at 37°C for 12–16 h with constant agitation (250 rpm), the culture was diluted 1:50, and the incubation continued under the same conditions up to A₆₀₀ 1.0–1.2. Bacteria were induced to overexpress recombinant protein by adding 0.5 mM isopropyl-thio-β-D-galactopyranoside (Sigma), followed by incubation at 18°C with constant agitation (250 rpm) for 12–16 h. Cells were harvested and frozen (–80°C) until needed. After thawing, lysis was achieved in the presence of buffer IMAC (20 mM Tris-Cl, pH 8.0, 0.5 M NaCl) plus 0.5% Triton X-100, 1 mM phenylmethylsulphonyl fluoride, 100 µg/ml DNase I, and lysozyme produced by the cells. After 10 min of lysis, the extract viscosity was reduced by six pulses of 30 s ultrasound with a Brandson 450 sonicator. Supernatants were centrifuged at 100,000 × *g* for 45 min and subjected to iminodiacetic acid metal affinity chromatography (IMAC chelating, Amersham Pharmacia Biotech, Little Chalfont, U.K.) Ni²⁺-charged. After applying the extract, the column was successively washed with buffer IMAC and 30 mM imidazole in buffer IMAC. The protein was eluted with 100 mM imidazole in the same buffer; dialyzed against 20 mM Tris-HCl, pH 8.8, 30 mM NaCl; and further purified by FPLC anionic exchange (MonoQ) applying a linear NaCl elution gradient. The activity peak was pooled and immediately desalted with a fast desalting column (Amersham Pharmacia Biotech). Purified proteins were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis under reducing conditions, stained with Coomassie blue R250 (Sigma), and quantitated with Kodak 1D 3.0 software using purified BSA as standard.

Preparation of 3-O-β-D-galactopyranosyl-D-arabinose and 3-O-β-D-galactopyranosyl-D-arabinitol

2,3,5,6,2',3',4',6'-Octa-O-acetyllactobionitrile was subjected to the Zemplen degradation method with 2 ml 0.5 M sodium methoxide in methanol affording a mixture of the four 3-O-β-D-galactopyranosyl-3'-D-arabinoses (α*p*, β*p*, α*f*, β*f*) that was characterized by ¹³C NMR. This mixture was reduced with NaBH₄ to give 3-O-β-D-galactopyranosyl-D-arabinitol that was also characterized by ¹³C NMR.

Preparation of 5-[3'-O-β-D-galactopyranosyl-D-gluco-pentitol-1'-yl]-tetrazole

The 5-[1',2',4',5',2'',3'',4'',6''-octa-O-benzoyl-3'-O-β-D-galactopyranosyl-D-gluco-pentitol-1'-yl]-tetrazole (115 mg) was dissolved in 0.5 M sodium methoxide in methanol (1 ml), and the solution was kept for 1 h at room temperature. After neutralization by passing through an Amberlite IR-120 (H⁺) plus resin column (3 ml) the product was eluted with water, lyophilized, and characterized by ¹H and ¹³C NMR spectroscopy.

HPAEC-PAD analysis

Analysis by HPAEC with pulsed amperometric detection (PAD) was performed using a Dionex DX-300 HPLC system equipped with a pulse amperometric detector. The following columns and conditions were used.

Condition 1. A CarboPac PA-100 ion exchange analytical column (4 × 250 mm) equipped with a guard column PA-100 (4 × 50 mm) was used with the following program: 50 mM NaAcO in 100 mM NaOH for 10 min followed by a linear gradient in 80 min from 50 to 500 mM NaAcO in 100 mM NaOH at a flow rate of 1.0 ml/min at room temperature.

Condition 2. A CarboPac PA-100 ion exchange analytical column (4 × 250 mm) equipped with a guard column PA-100 (4 × 50 mm) was used with the following program: a linear gradient over 80 min from 100 to 500 mM NaAcO in 100 mM NaOH at a flow rate of 1.0 ml/min at room temperature.

Condition 3. A CarboPac PA-10 ion exchange analytical column (4 × 250 mm) equipped with a guard column PA-10 (4 × 50 mm) was eluted isocratically with 100 mM NaOH, 50 mM AcONa at a flow rate of 1.0 ml/min at room temperature.

Condition 4. A CarboPac PA-10 ion exchange analytical column (4 × 250 mm) equipped with a guard column PA-10 (4 × 50 mm) was used with the following program: 50 mM AcONa in 100 mM NaOH for 10 min followed by a gradient elution over 20 min from 50 to 100 mM AcONa in 100 mM NaOH at a flow rate of 1.0 ml/min at room temperature.

Condition 5. A CarboPac PA-10 ion exchange analytical column (4 × 250 mm) equipped with a guard column PA-10 (4 × 50 mm) was used isocratically with 60 mM NaOH at a flow rate of 1.0 ml/min at room temperature.

Enzyme kinetics

Reaction mixtures of 20 μ l containing 20 mM Tris buffer, pH 7, 30 mM NaCl, 1 mM 3'-sialyllactose as donor and 1 mM acceptor substrate were incubated with 40 ng purified trans-sialidase for different times (0, 15, 30, and 60 min) at 25°C. After incubation, reaction mixtures were diluted with 100 μ l deionized water and frozen. Sialylation of acceptors was calculated as the percentage of the sialylated product obtained over the total amount of sialic acid (free or linked to a saccharide). For K_m calculations, different concentrations of substrate were incubated for 15 min at 25°C as before. Samples were then diluted 36 times, and 20 μ l of each were analyzed by HPAEC. Under the incubation conditions free sialic acid was not detected. The extent of sialylation of substrates was calculated from the decrease in the concentration of 3'-sialyllactose and K_m values were obtained graphically by the Lineweaver-Burk method. The rate of reaction was calculated considering the percentage of sialylated product over the total amount of sialic acid (1 mM) and a reaction time of 15 min.

Inhibition of sialylation of N-acetyllactosamine

Reaction mixtures of 20 μ l containing buffer 20 mM Tris-HCl, pH 7, 30 mM NaCl, 1 mM 3'-sialyllactose, 1 mM N-acetyllactosamine, and different concentrations of inhibitors were incubated with purified trans-sialidase for 30 min at room temperature. Samples were then diluted 36 times with deionized water and analyzed by HPAEC. Inhibition was calculated from the percentages of 3'-sialyllactosamine obtained with and without inhibitor.

Sialyllactobionic acid as a donor

Sialyllactobionic acid was prepared by incubation of 20 μ l of an aqueous solution of fetuin (Sigma F2379) containing 10 mM sialic acid linked to protein, 20 μ l 10 mM lactobionic acid, and 3 μ l trans-sialidase (60 ng) in a total volume of 200 μ l 20 mM Tris, 30 mM NaCl, pH 7, buffer for 1 h at 25°C. Reaction mixture was diluted with 2.3 ml water and applied to a Sephadex-25 P-10 column (Amersham Pharmacia). Elution was performed with water, the first 6 ml were discharged, and the sialyllactobionic acid was eluted with another 6 ml water. After lyophilization, the sialyllactobionic acid was separated by HPAEC and decationized through an Amberlite IR-120 (H⁺) column that was eluted with water and lyophilized. To confirm that the product contained sialic acid, it was hydrolyzed with 0.1 M HCl for 30 min at 80°C, and the hydrolyzate was analyzed by HPAEC. Purified sialyllactobionic acid was used as sialic acid donor in an incubation containing 1 mM lactose as acceptor and 40 ng trans-sialidase for 1 h at 25°C.

3'-Sialyllactitol and 3'-sialyl-N-acetyllactosamine as donors

3'-Sialyllactitol and 3'-sialyl-N-acetyl-lactosamine were recovered from the TcTS incubations by collecting the corresponding fractions from HPAEC. Solutions were then decationized with an Amberlite IR-120(H⁺) column (1 ml) eluting with 5 ml water. Different concentrations of

the acidic compounds were incubated with 2 μ l 10 mM lactose and 40 ng trans-sialidase in a total volume of 20 μ l 20 mM Tris-HCl (pH 7) buffer, 30 mM NaCl, for 30 min at 25°C. Reaction mixtures were diluted and analyzed by HPAEC.

Inhibition of sialylation of lactose by lactobionic acid

A reaction mixture containing 20 μ l lactose (10 mM), 20 μ l fetuin (10 mM in sialic acid), and 60 ng trans-sialidase was incubated with or without 20 μ l lactobionic acid (10 mM) in a total volume of 200 μ l Tris (pH 7) buffer for 1 h at 25°C. After filtration through a Sephadex G-25 P-10 column, the included volume was analyzed by HPAEC-PAD.

Cell invasion experiments

Vero cells were plated in a 24-well plate (10⁵ cells/well) with a sterile 12-mm-diameter glass coverslip in 0.5 ml 10% FBS-MEM and used after 24 h of growth. Before infection, medium was removed and cells were washed twice with PBS; 0.2% BSA-MEM was added in absence or presence of 10 mM lactitol or 10 mM lactose (Sigma) as indicated in each experiment. Trypomastigotes (200 parasites/cell) were incubated for 20 h with cells at 37°C in 5% CO₂. Similar results were obtained with 3 h of contact between cells and trypomastigotes, but the percentage of infection was lower. When indicated, trypanosomes were sialylated before infection with 3 μ g recombinant trans-sialidase and 1 mM sialyllactose as donor of sialic acid for 30 min. After removing the parasites, the cells were washed with PBS and maintained with 3% FBS-MEM. After 72 h of infection, coverslips were washed twice with PBS; the cells were fixed and stained 5 min with 250 μ l of May-Grünwald-Giemsa stain; and then 250 μ l water was added. Reagents were removed, and 600 μ l Giemsa (1:4 in water) was added in each well. After 5 min, coverslips were washed twice with water and mounted in slides using DPX mountant for histology (Fluka, St. Louis, MO). The number of infected cells was counted in at least 500 cells at 400 \times . Each experiment was done in triplicate. *p*-Values were calculated using unpaired two-tailed *t*-test (*p* < 0.05). Results were expressed as the mean \pm SE of the percentage of infected cells normalized to the values obtained with control trypomastigotes, which were considered 100%.

Sialic acid measurements

Trypomastigotes collected from each of the indicated experiments were stored frozen at -70°C until analysis. Total amount of sialic acid in parasites was determined after hydrolysis in 0.1 M HCl for 1 h at 80°C using the thiobarbituric acid method and HPLC analysis (Powell and Hart, 1986). Results are expressed as pmol of sialic acid per 10⁶ parasites.

Acknowledgments

We thank B. Cazzulo, L. Sferco, and A. Chidichimo for technical assistance with parasite and cell cultures; A. Meras for help with protein purification and sialic acid determination; and G. Gotz for reading the manuscript.

This work was supported by grants from the Human Frontier Science Program and the Agencia Nacional de Promoción Científica y Tecnológica (Argentina). The research from A.C.C.F. was supported in part by an International Research Scholars Grant from the Howard Hughes Medical Institute. G.P. is a research fellow and A.C.C.F. and R.M.L. are researchers from the National Research Council (CONICET), Argentina.

Abbreviations

BSA, bovine serum albumin; HPAEC, high pH anion-exchange chromatography; HPLC, high-performance liquid chromatography; MEM, minimum essential medium; NMR, nuclear magnetic resonance; PAD, pulse amperometric detection; PBS, phosphate buffered saline; TcTS, *Trypanosoma cruzi* trans-sialidase.

References

- Acosta-Serrano, A., Almeida, I.C., Freitas-Junior, L.H., Yoshida, N., and Schenkman, S. (2001) The mucin-like glycoprotein super-family of *Trypanosoma cruzi*: structure and biological roles. *Mol. Biochem. Parasitol.*, **114**, 143–150.
- Agusti, R., Couto, A.S., Campetella, O.E., Frasch, A.C., and de Lederkremer, R.M. (1997) The trans-sialidase of *Trypanosoma cruzi* is anchored by two different lipids. *Glycobiology*, **7**, 731–735.
- Agusti, R., Couto, A.S., Campetella, O., Frasch, A.C., and de Lederkremer, R.M. (1998) Structure of the glycosylphosphatidylinositol-anchor of the trans-sialidase from *Trypanosoma cruzi* metacyclic trypomastigote forms. *Mol. Biochem. Parasitol.*, **97**, 123–131.
- Amaya, M.F., Buschiazzi, A., Nguyen, T., and Alzari, P.M. (2003) The high resolution structures of free and inhibitor-bound *Trypanosoma rangeli* sialidase and its comparison with *T. cruzi* trans-sialidase. *J. Mol. Biol.*, **325**, 773–784.
- Belen Carrillo, M., Gao, W., Herrera, M., Alroy, J., Moore, J.B., Beverley, S.M., and Pereira, M.A. (2000) Heterologous expression of *Trypanosoma cruzi* trans-sialidase in *Leishmania major* enhances virulence. *Infect. Immun.*, **68**, 2728–2734.
- Buschiazzi, A., Campetella, O., and Frasch, A.C. (1997) *Trypanosoma rangeli* sialidase: cloning, expression and similarity to *T. cruzi* trans-sialidase. *Glycobiology*, **7**, 1167–1173.
- Buschiazzi, A., Tavares, G.A., Campetella, O., Spinelli, S., Cremona, M.L., Paris, G., Amaya, M.F., Frasch, A.C., and Alzari, P.M. (2000) Structural basis of sialyltransferase activity in trypanosomal sialidases. *EMBO J.*, **19**, 16–24.
- Buschiazzi, A., Amaya, M.F., Cremona, M.L., Frasch, A.C., and Alzari, P.M. (2002) The crystal structure and mode of action of trans-sialidase, a key enzyme in *Trypanosoma cruzi* pathogenesis. *Mol. Cell*, **10**, 757–768.
- Cerecetto, H. and Gonzalez, M. (2002) Chemotherapy of Chagas' disease: status and new developments. *Curr. Top. Med. Chem.*, **2**, 1187–1213.
- D'Accorso, N.B.D., Zuazo, B.N., and Thiel, I.M.E. (1988) Synthesis and spectra of some octa-*O*-benzoylaldobionitriles. *Carbohydr. Res.*, **172**, 147–155.
- Frasch, A.C. (2000) Functional diversity in the trans-sialidase and mucin families in *Trypanosoma cruzi*. *Parasitol. Today*, **16**, 282–286.
- Fujimura, A.E., Kinoshita, S.S., Pereira-Chioccola, V.L., and Rodrigues, M.M. (2001) DNA sequences encoding CD4⁺ and CD8⁺ T-cell epitopes are important for efficient protective immunity induced by DNA vaccination with a *Trypanosoma cruzi* gene. *Infect. Immun.*, **69**, 5477–5486.
- Katae, M., Miyahira, Y., Takeda, K., Matsuda, H., Yagita, H., Okumura, K., Takeuchi, T., Kamiyama, T., Ohwada, A., Fukuchi, Y., and Aoki, T. (2002) Co-administration of an interleukin-12 gene and a *Trypanosoma cruzi* gene improves vaccine efficacy. *Infect. Immun.*, **70**, 4833–4840.
- Kirchhoff, L.V. (2003) Changing epidemiology and approaches to therapy for Chagas disease. *Curr. Infect. Dis. Rep.*, **5**, 59–65.
- Leguizamon, M.S., Campetella, O.E., Gonzalez Cappa, S.M., and Frasch, A.C. (1994) Mice infected with *Trypanosoma cruzi* produce antibodies against the enzymatic domain of trans-sialidase that inhibit its activity. *Infect. Immun.*, **62**, 3441–3446.
- Leguizamon, M.S., Mocetti, E., Garcia Rivello, H., Argibay, P., and Campetella, O. (1999) Trans-sialidase from *Trypanosoma cruzi* induces apoptosis in cells from the immune system *in vivo*. *J. Infect. Dis.*, **180**, 1398–1402.
- Lopez, M., Huynh, C., Andrade, L.O., Pypaert, M., and Andrews, N.W. (2002) Role for sialic acid in the formation of tight lysosome-derived vacuoles during *Trypanosoma cruzi* invasion. *Mol. Biochem. Parasitol.*, **119**, 141–145.
- Montagna, G., Cremona, M.L., Paris, G., Amaya, M.F., Buschiazzi, A., Alzari, P.M., and Frasch, A.C. (2002) The trans-sialidase from the african trypanosome *Trypanosoma brucei*. *Eur. J. Biochem.*, **269**, 2941–2950.
- Mucci, J., Hidalgo, A., Mocetti, E., Argibay, P.F., Leguizamon, M.S., and Campetella, O. (2002) Thymocyte depletion in *Trypanosoma cruzi* infection is mediated by trans-sialidase-induced apoptosis on nurse cells complex. *Proc. Natl Acad. Sci. USA*, **99**, 3896–3901.
- Okazaki, S., Suwa, K., Hamasu, Y., and Sumi, N. (1994) Single dose toxicity studies of lactitol (NS-4) in mice and rats. *J. Toxicol. Sci.*, **19**(suppl 3), 295–299.
- Othegui, L.C., Fascio, M.L., Martins Alho, M.A., D'Accorso, N.B.D., and Thiel, I.M.E. (1990) Synthesis and spectra for some 5-[octa-*O*-benzoyl-3'-*O*-*D*-hexopyranosyl-*D*-gluco-pentitol-1'-yl] tetrazoles. *Anal. Asoc. Quim. Argentina*, **78**, 65–74.
- Paris, G., Cremona, M.L., Amaya, M.F., Buschiazzi, A., Giambiagi, S., Frasch, A.C., and Alzari, P.M. (2001) Probing molecular function of trypanosomal sialidases: single point mutations can change substrate specificity and increase hydrolytic activity. *Glycobiology*, **11**, 305–311.
- Parodi, A.J., Pollevick, G.D., Mautner, M., Buschiazzi, A., Sanchez, D.O., and Frasch, A.C. (1992) Identification of the gene(s) coding for the trans-sialidase of *Trypanosoma cruzi*. *EMBO J.*, **11**, 1705–1710.
- Pitcovsky, T.A., Buscaglia, C.A., Mucci, J., and Campetella, O. (2002) A functional network of intramolecular cross-reacting epitopes delays the elicitation of neutralizing antibodies to *Trypanosoma cruzi* trans-sialidase. *J. Infect. Dis.*, **186**, 397–404.
- Pontes de Carvalho, L.C., Tomlinson, S., Vandekerckhove, F., Bienen, E.J., Clarkson, A.B., Jiang, M.S., Hart, G.W., and Nussenzweig, V. (1993) Characterization of a novel trans-sialidase of *Trypanosoma brucei* procyclic trypomastigotes and identification of procyclin as the main sialic acid acceptor. *J. Exp. Med.*, **177**, 465–474.
- Powell, L.D. and Hart, G.W. (1986) Quantitation of picomole levels of N-acetyl- and N-glycolylneuraminic acids by a HPLC-adaptation of the thiobarbituric acid assay. *Anal. Biochem.*, **157**, 179–185.
- Ribeiro, M., Pereira-Chioccola, V.L., Eichinger, D., Rodrigues, M.M., and Schenkman, S. (1997) Temperature differences for trans-glycosylation and hydrolysis reaction reveal an acceptor binding site in the catalytic mechanism of *Trypanosoma cruzi* trans-sialidase. *Glycobiology*, **7**, 1237–1246.
- Salto, M.L., Gallo-Rodriguez, C., Lima, C., and de Lederkremer, R.M. (2000) Separation of Galβ1→XGlcNAc and Galβ1→XGlcNAc (X = 3, 4, and 6) as the alditols by high-pH anion-exchange chromatography and thin-layer chromatography: characterization of mucins from *Trypanosoma cruzi*. *Anal. Biochem.*, **279**, 79–84.
- Schenkman, S., Jiang, M.S., Hart, G.W., and Nussenzweig, V. (1991) A novel cell surface trans-sialidase of *Trypanosoma cruzi* generates a stage-specific epitope required for invasion of mammalian cells. *Cell*, **65**, 1117–1125.
- Schenkman, S., Ferguson, M.A., Heise, N., de Almeida, M.L., Mortara, R.A., and Yoshida, N. (1993) Mucin-like glycoproteins linked to the membrane by glycosylphosphatidylinositol anchor are the major acceptors of sialic acid in a reaction catalyzed by trans-sialidase in metacyclic forms of *Trypanosoma cruzi*. *Mol. Biochem. Parasitol.*, **59**, 293–303.
- Schenkman, S., Eichinger, D., Pereira, M.E., and Nussenzweig, V. (1994) Structural and functional properties of *Trypanosoma* trans-sialidase. *Annu. Rev. Microbiol.*, **48**, 499–523.

- Scudder, P., Doom, J.P., Chuenkova, M., Manger, I.D., and Pereira, M.E. (1993) Enzymatic characterization of beta-D-galactoside alpha 2,3-trans-sialidase from *Trypanosoma cruzi*. *J. Biol. Chem.*, **268**, 9886–9891.
- Tiralongo, E., Schrader, S., Lange, H., Lemke, H., Tiralongo, J., and Schauer, R. (2003) Two trans-sialidase forms with different sialic acid transfer and sialidase activities from *Trypanosoma congolense*. *J. Biol. Chem.*, **278**, 23301–23310.
- Tomlinson, S., Pontes de Carvalho, L.C., Vandekerckhove, F., and Nussenzweig, V. (1994) Role of sialic acid in the resistance of *Trypanosoma cruzi* trypomastigotes to complement. *J. Immunol.*, **153**, 3141–3147.
- Urbina, J.A. and Docampo, R. (2003) Specific chemotherapy of Chagas disease: controversies and advances. *Trends Parasitol.*, **19**, 495–501.
- Vandekerckhove, F., Schenkman, S., Pontes de Carvalho, L., Tomlinson, S., Kiso, M., Yoshida, M., Hasegawa, A., and Nussenzweig, V. (1992) Substrate specificity of the *Trypanosoma cruzi* trans-sialidase. *Glycobiology*, **2**, 541–548.
- Watts, A.G., Damager, I., Amaya, M.L., Buschiazzi, A., Alzari, P., Frasch, A.C., and Withers, S.G. (2003) *Trypanosoma cruzi* trans-sialidase operates through a covalent sialyl-enzyme intermediate: tyrosine is the catalytic nucleophile. *J. Am. Chem. Soc.*, **125**, 7532–7533.
- Wizel, B., Garg, N., and Tarleton, R.L. (1998) Vaccination with trypomastigote surface antigen 1-encoding plasmid DNA confers protection against lethal *Trypanosoma cruzi* infection. *Infect. Immun.*, **66**, 5073–5081.
- Zingales, B., Pereira, M.E., Almeida, K.A., Umezawa, E.S., Nehme, N.S., Oliveira, R.P., Macedo, A., and Souto, R.P. (1997) Biological parameters and molecular markers of clone CL Brener—the reference organism of the *Trypanosoma cruzi* genome project. *Mem. Inst. Oswaldo Cruz*, **92**, 811–814.