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Invasion of *Trypanosoma cruzi* into host cells is impaired by *N*-propionylmannosamine and other *N*-acylmannosamines

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Abstract The etiologic agent of Chagas' disease, *Trypanosoma cruzi*, is widely distributed in South America, affecting millions of people with thousands of deaths every year. Adherence of the infectious trypomastigote to host cells is mediated by sialic acid. *T. cruzi* cannot synthesize sialic acids on their own but cleave them from the host cells and link them to glycans on the surface of the parasites using the trans-sialidase, a GPI-anchored enzyme. The infectivity of the protozoan parasites strongly depends on the activity of this enzyme. In this report, we investigated whether the transfer of sialic acids from the host to the parasites can be attenuated using novel sialic acid precursors. The cell line 86-HG-39 was infected with *T. cruzi* and treated with defined *N*-acylmannosamine analogues bearing an elongated *N*-acyl side-chain. By treatment of these cells the number of *T.cruzi* infected cell was reduced up to 60%. We also showed that the activity of the bacterial sialidase C was reduced with N-glycan substrates with elongated *N*-acyl side chains of the terminal sialic acids. The affinity of this sialidase decreased with the length of the *N*-acyl side-chain. The data presented suggest that *N*-acyl modified sialic acid precursors can change the transfer of sialic acids leading to modification of infection. Since the chemotherapy of this disease is inefficient and afflicted by side effects, the need of effective drugs is lasting. These findings propose a new path to prevent the dissemination of *T. cruzi* in the human hosts. These compounds or further modified analogues might be a basis for the search of new agents against Chagas' disease.

Keywords *T. cruzi* · Trans-sialidase-inhibition · *N*-propionyl-mannosamine · *N*-acyl side chain · Engineered sialic acids

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Introduction

Trypanosoma cruzi is the causative agent of Chagas' disease. In South America nearly 100 million people are at risk of infection, with 16–18 millions infected patients and several thousands deaths per year [1]. Because of the long host-parasite history *T. cruzi* is well adapted to humans and manages to survive for more than decades in the host despite a pronounced immune response. *T. cruzi* is transmitted by blood sucking bugs, which are only endemic in Central and South America. Although the affected countries attempt great efforts in the Southern Cone One co-operation targeting the insect vector it is not possible to avert numerous new infections per year caused by sylvatic populations of the insect vector [2].

Mammalian hosts are infected by trypomastigotes of *T. cruzi*, that spread through the blood stream in the body and invade almost every tissue. The trypomastigotes adhere to

the surface of host cells and induce phagocytosis [3, 4], which is mediated by sugar residues of the respective glycoconjugates, especially by sialic acids on the parasite's surface [5–7]. Trypomastigotes evade lysis by escaping the phagosome and multiply as amastigotes in the cytosol.

Expression of sialic acids connected to glycoproteins and glycolipids play an important role during the regulation of numerous processes of mammalian cells [8, 9]. Sialic acid protects cells from invasion of pathogens by masking binding sites on host galactose residues [8]. Unfortunately, many bacterial pathogens express sialidases to unmask the binding sites of the host. Conversely, sialic acid is a shield not only against infections but is also a ligand for viruses *e.g.* the hemagglutinin of influenza A virus, which is crucial for invasion of the pathogen into cells [10]. Moreover, it was previously shown that the length of the *N*-acyl side chain of host's sialic acid was able to modulate virus invasions [11, 12].

A hallmark in *T. cruzi* research was the finding that *T. cruzi* is not able to synthesize its own sialic acid, but scavenges it from host sialoglycoconjugates by a novel type of sialidase, the trans-sialidase (TS) [7, 13–15]. This unique GPI-anchored enzyme splits off sialic acid from the host sialoconjugates and transfers it to mucins on the parasite's surface. It catalyzes the (α2-3) transfer of host's sialic acid to O-glycan type glycoproteins of the parasite. All known intracellular sialylation processes known until recently need the activation of sialic acid to CMP-sialic acid for its transfer. The endowment of *T. cruzi's* surface with sialic acid promotes adhesion of trypomastygotes by interacting with sialyl epitopes of the host cell surface [16]. Recently, it has been reported that sialic acid on the parasites surface attaches to sialic acid-binding Ig-like lectin (Siglec)-E, which leads to immune suppression in dendritic cells [17]. Furthermore, sialic acid protects the parasite against complement-induced destruction and it is essential for the formation of chronic inflammation [18]. Due to its pivotal role in Chagas' disease the TS is a promising target in attempts to search for novel approaches of treatment.

Very recently, inhibition of *T. cruzi* infection has been achieved by the use of 3-fluorosialyl fluoride, which interferes with the glycosyl-enzyme intermediate [19]. In our study we focused on the role of the *N*-acyl side chain of sialic acid to inhibit TS activity. In earlier studies we could show that the *N*-acyl side chain can become biochemically engineered by treatment of mammalian cells with *N*-propionyl-mannosamine (and other *N*-acylhomologues) leading to the respective sialic acids. This novel kind of biochemical engineering influences defined biological activities [20]. As a consequence, we could demonstrate that cell invasion of *T. cruzi* is inhibited by treatment of host cells with *N*-acyl mannosamine analogues suggesting an alternative path to prevent full-blown development of *T. cruzi* in the human host.

Materials and methods

Synthesis of *N*-acylmannosamine derivates

Derivates were synthesised as described earlier [21]. In brief, D-mannosamine hydrochloride was suspended in methanol and subsequently in ice cold Na-methylate/methanol and the corresponding carbonic acid anhydride was added. After stirring for 2 h the solution was dried in a vacuum evaporator. The raw product was purified by column chromatography on silica gel 60 (Merck, Germany) and characterisation was carried out by ^{1}H NMR spectroscopy. *N*-Acylmannosamine derivates were stored at 4°C and dissolved in PBS as a stock solution of 100–200 mM shortly before addition to the cell culture. The different mannosamines are shown in Fig. 1.

Parasites and cells

T. cruzi strain Tulahuen (WHO reference strain M/HOM/CH/00/Tulahuen C2) is maintained in culture by weekly inoculation of adherent human myoblastoma cell line 86-HG-39 cells with infectious trypomastigotes drawn from the supernatants of previously infected cells using RPMI 1640 medium (PAA Laboratories, Germany) supplemented with 5% FCS (Biochrom AG, Germany), 2 mM glutamine and 1% gentamycine (PAA Laboratories).

CHO-K1 cells [22] were cultured in DMEM medium (PAA Laboratories) containing 10% FCS (Biochrom) to which 2 mM glutamine and penicillin/streptomycin (100 U and 0.1 mg/ml) were added (PAA Laboratories). *N*-Acyl-D-mannosamine derivatives were added to a final concentration of 5 mM (0.5 mM for peracetylated ManNprop). *N*-Acyl-D-mannosamine derivatives were supplemented in fresh media after culturing the cells for 4 days. CHO cells were harvested after a week of culture.

Isolation of membrane proteins

After harvesting and washing 1×10^{8} cells in phosphate buffered saline, cell lysis was performed using a 10 ml Potter-Elvehjem homogenizer in a buffer consisting of 1 mM $NaHCO_3$, 150 mM KCl, 2 mM $CaCl_2$, pH 7.6 and protease inhibitors (complete EDTA-free, Roche, Germany). The cell lysate was centrifuged at 2,000 rpm for 15 min; the pellet was discarded. The supernatant was centrifuged at 20,000 rpm for 30 min and the pellet, containing the membrane glycoproteins, was collected and redissolved in water, methanol and chloroform (3:8:4) in order to separate lipids from membrane glycoproteins. After 10 min sonication and periodic shaking, the separation was achieved by centrifugation at 12,000 rpm for 5 min. Finally, the pellet was resuspended in 50 mM phosphate buffer pH 7.

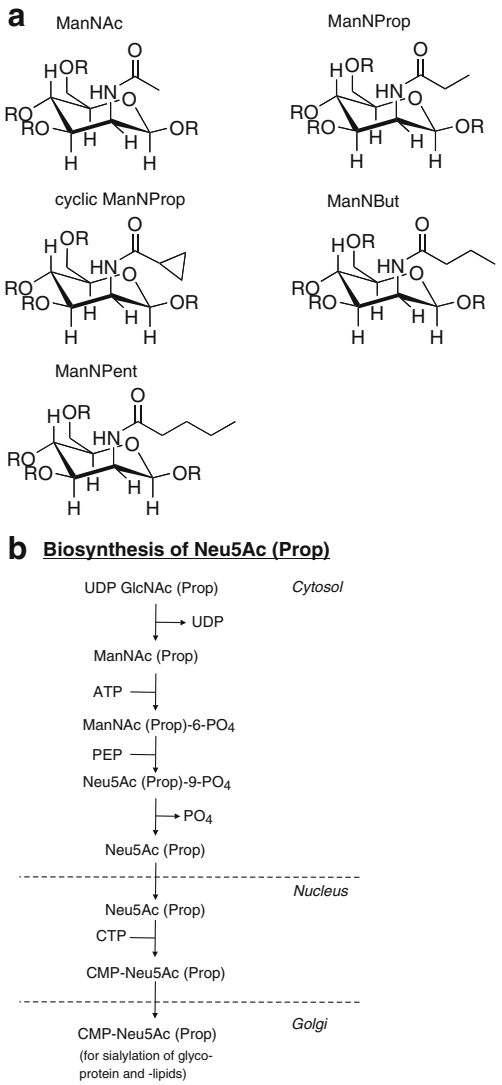

Fig. 1 Schematic presentation of modified *N*-acyl mannosamines and metabolism. The structure of *N*-acyl D-mannosamine is elongated with one (*N*-propanoyl-D-mannosamine; ManNProp), two (*N*-butanoyl-D-mannosamine; ManNBut) or three (*N*-pentanoyl-D-mannosamine; ManNPent) acyl chains. For peracetylated ManNProp *R* = Ac, for all other analogues *R* = H (**a**). The deacetylated analogues are metabolised like common ManNAc resulting in modified CMP-Neu5Ac (**b**)

Release and isolation of N-glycans

Membrane glycoproteins, dissolved in 500 µl 50 mM phosphate buffer pH 7, were digested with 10 µg trypsin (Sigma-Aldrich, Germany) for 16 h at 37°C. Samples were then digested with 1 U peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase F (Roche Applied Science, IN) [23–25] for 16 h at 37°C. N-Glycans were isolated using reversed-phase C18 cartridges (Grace Davison Discovery Sciences, Deerfield, IL) and desalted on graphitized carbon columns (Grace Davison Discovery Sciences, Deerfield, IL) [26].

2AB-labeling of glycans

N-Glycans were labelled with 0.35 M 2-aminobenzamide (2AB) /1 M sodium cyanoborohydride in acetic acid-dimethylsulfoxide (3:7, v/v) (8 µl) for 2 h at 65°C [27–29]. The excess of 2AB-reagent was removed by paper chromatography using two times *n*-butanol/ethanol/H_2O (4:1:1) as mobile-phase. N-Glycans were eluted with 3×500 µl H_2O and finally lyophilized.

2AB-HPLC profiling

HPLC profiling of the sialylated 2-AB N-glycans was performed on a Shodex Asahi-Pak-NH2 P-50 E4 5µ (250×4.6 mm, Shodex, Germany) column using a Dionex Ultimate 3000 HPLC system, equipped with a Dionex fluorescence detector RF2000 ($\lambda_{exc.max}$=330 nm, $\lambda_{em.max}$=420 nm). Elutions were performed at a flow rate of 0.8 ml/min at 50°C using the following eluents: A, 2% acetic acid and 1% tetrahydrofuran in acetonitrile; B, 5% acetic acid, 1% tetrahydrofuran, 3% triethylamine in water. A linear gradient 0–82 min with 30–95% B was applied.

Sialidase digestion

N-Glycans

Each 2AB-labelled N-glycan pool was incubated with 30 mU (α2-3,6) sialidase C (recombinant from *Clostridium perfringens*, expressed in *E. coli*, Prozyme, Germany) at 37°C in 50 mM potassium phosphate, pH 6.0, for 15 min to 16 h [30]. Samples were subsequently inhibited at 95°C for 5 min and desalted on graphitized carbon columns (Grace Davison Discovery Sciences, Deerfield, IL).

86-HG-39 cells

A monolayer of 86-HG-39 cells was incubated in serum free RPMI medium supplemented with 8 µg/ml sialidase C for 60 min at 37°C. Medium was discarded and cells were washed with fresh medium supplemented with 10% FCS before being infected with trypomastigotes in a MOI of 1.

Trypomastigotes

1×10^6 Trypomastigotes were incubated in 1 ml serum free RPMI medium supplemented with 8 µg/ml sialidase C for 60 min at 37°C. Parasites were subsequently centrifuged and washed with fresh medium supplemented with 10% FCS.

To avoid effects of re-expression of sialic acids on the host cells on infection rate of sialidase treated host cells and parasites, remaining trypomastigotes were removed after 12 h and cells were cultured for additional 3 days in fresh medium supplemented with 10% FCS.

Assessment of parasitic load

5×10^3 86-HG-39 cells were cultured in eight well Lab-Tek chamber slides (Nunc, Thermo Fisher Scientific, Langenselbold, Germany) for 24 h before treatment to assure adherence. Cells were then further cultured for 3 days to a monolayer in the presence of five different *N*-acylmannosamine derivates in a final concentration of 5 mM *N*-propionylmannosamine (ManNProp), *N*-butanoylmannosamine (ManNBut), *N*-pentanoylmannosamine (ManNPent), *N*-cyclopropylcarbonylmannosamine (cyclic ManNProp) and 0.5 mM peracetylated ManNProp, respectively.

After exchange of medium, again supplemented with the different *N*-acylmannosamine derivatives, cells were infected with $3–5\times10^4$ *T. cruzi* and incubated for additional 3 days. Cells were subsequently fixed with 2% paraformaldehyde and labelled with DAPI, a dye for nuclear fluorescent staining. Parasitic load was assessed by counting the number of infected cells by fluorescent microscopy.

Statistical analysis Results are presented as the mean plus standard deviations. Statistical analysis was generally performed with the unpaired Student's *t* test using the prism software (Graph Pad Software, San Diego, CA).

Results

Biosynthesis of sialic acids starting with *N*-acetylmannosamine analogues

Previous studies carried out in eukaryotic cells demonstrated that *N*-acyl modified mannosamines (Fig. 1a) are metabolized to the respective CMP-sialic acids by the promiscuous enzymes metabolizing the physiological *N*-acetylmannosamine [31]. The different sialic acids are incorporated into glycoconjugates of membranes or the serum [20, 32] (Fig. 1b).

Sialidase treatment of parasites and/or host cells decreases infection of host cells

Surface sialic acid is involved in the continuation of the cell cycle of the protozoan parasite *T. cruzi*. We treated either host cells or parasites or both with sialidase of *C. perfringens*, respectively (Fig. 2). The resulting infection rate of the host cells was determined after 3 days by fixing the cells. Subsequently, a nuclear staining was performed to allow counting of cells with intracellular amastigotes. Treatment of host cells with sialidase C revealed a 30% decreased infection rate in comparison to untreated cells. The inhibition of invasion of host cells by *T. cruzi* trypomastigotes was more efficient by treating trypomastigotes with sialidase (50% inhibition). However, the highest rate was with 70% inhibition achieved by treating both the host cells and the trypomastigotes. In addition, 86-HG-39 cells were incubated with trypomastigotes in the presence of lactose, which acts as a competitive acceptor of sialic acids for *T. cruzi* TS resulting in remarkable less sialylation of *T. cruzi's* mucins [33]. This treatment led to a 30% decreased infection, comparable to the treatment of host cells with sialidase.

N-Glycans with *N*-acyl modified sialic acids are more resistant towards sialidase digestion

We tested *in vitro* the resistance of the *N*-acyl modified neuraminic acids with bacterial sialidase C using *N*-glycans isolated from CHO cells. CHO cells were separately incubated with ManNAc and three *N*-acyl analogues (cyclic ManNProp, ManNBut and ManNPent). Cells were harvested and total membrane fractions were isolated from the cell lysate. The rate of incorporation of the modified sialic acids is dependent on the cell type. For CHO cells, it was about 70–90%, whereas in earlier work, it was only ranging from 18–35% using MDCK II cells [12]. In both cell types,

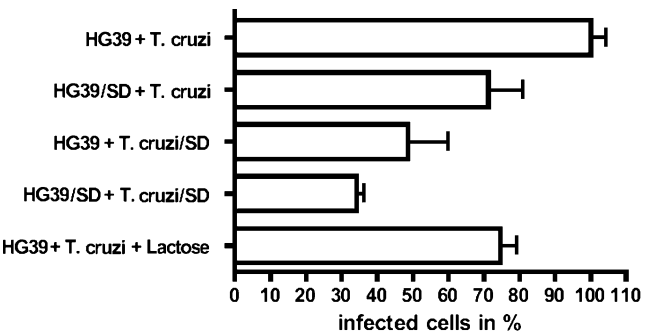

Fig. 2 Treatment of host cells and/or *T. cruzi* trypomastigotes with sialidase inhibits invasion of the parasites. A monolayer of 86-HG-39 cells were left either untreated or incubated with 8 μg/ml sialidase of *C. perfringens* for 1 h at 37°C. Subsequently, cells were infected with trypomastigotes in a MOI of 1, which were either untreated or sialidase (SD) treated like the 86-HG-39 cells. In an additional sample, the cells were infected with $3\text{-}5\times10^4$ *T. cruzi* in the presence of 1 mM Lactose which is a competitive acceptor for sialic acids transferred by trans sialidase. After 3 days of infection cells were fixed with 2% paraformaldehyde and labelled with DAPI, a dye for nuclear fluorescent staining. Parasitic load was assessed by counting percentages of infected cells in fluorescent microscopy

the rate of incorporation was highest with the slightest modifications *i.e.* propionyl instead of acetyl group. N-Glycans of membrane glycoproteins were released using PNGase F followed by their isolation using reversed-phase and graphite cartridges. After being labelled with 2AB, N-glycans were fractionated according to charge by HPLC on an Asahi-Pak-NH_2 column. The monosialylated N-glycan fraction was incubated with sialidase C during 0 to 16 h. The rate of desialylation was estimated by HPLC (Fig. 3). Natural as well as biochemically engineered N-glycans with ManNProp or cyclic ManNProp as precursors were similarly digested by sialidase C, namely 60% to 70% of the sialic acids had been cleaved after 16 h. N-Glycans bearing longer *N*-acyl side-chains of the respective sialic acids showed a higher resistance towards sialidase as 15% only were digested after 16 h. *In vitro* the resistance of N-glycans towards bacterial sialidase C depends on the length of the *N*-acyl side of sialic acids.

N-Acylmannosamine analogues inhibit *T. Cruzi* infection of host cells

Further on, we addressed the question if the modification of sialic acids has an impact on *T. cruzi* cell invasion. Figure 4a shows a reduced number of amastigotes of *T. cruzi* in the host cells when the media have been supplemented with peracetylated ManNProp compared to control cells. The inhibition of infection was obvious using peracetylated ManNProp but not significant (p=0,0811). The same accounts for another analogue namely cyclic ManNProp (p=0,115) (Fig. 4b). However, incubation of host cells in medium supplemented with ManNProp (p=0,0477), ManNPent (p=0,026) and ManNBut (p=0,0279) caused significant decrease of infected host cells (Fig. 4b).

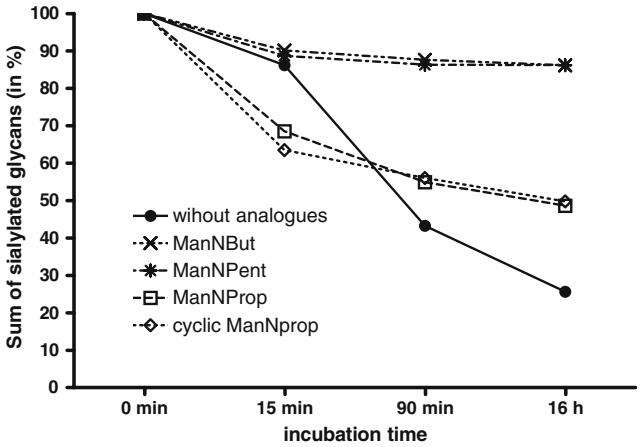

Fig. 3 Sialidase C resistance measured on monosialylated N-glycans bearing native or modified sialic acids. Membrane glycoprotein N-glycans were isolated from CHO cells that had been supplemented with 5 mM ManNProp, 5 mM cyclic ManNProp or 5 mM ManNBut. N-glycans, labelled with 2AB, were fractionated by HPLC on Asahi-Pak-NH2 according to charge. The monosialylated N-glycan fraction was collected and digested with sialidase C. The quantity of monosialylated N-glycans remaining after the digestion was estimated by HPLC. Graph shows the average of two independent experiments

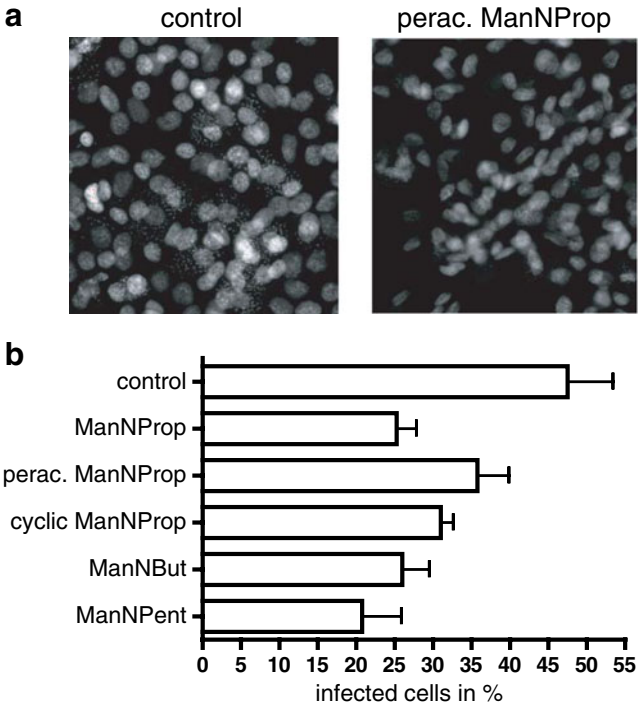

Fig. 4 Modification of glycans expressed on the surface of the host cells by different sugar derivates leads to significant decreased infection rate of 86-HG-39 cells by trypomastigotes of *T. cruzi*. 86-HG-39 cells were cultured for 24 h before treatment to assure adherence. Medium was completely discarded and replaced by medium supplemented with the *N*-acyl-D-mannosamine derivates ManNProp, cyclic ManNProp, ManNBut, ManNPent at a final concentration of 5 mM and peracetylated ManNProp at a final concentration of 0.5 mM, respectively and further cultured for 3 days. As control, cells were left untreated. Medium was again discarded and cells were infected with $3–5\times10^4$ *T. cruzi* in the presence of freshly added derivates and incubated for additional 3 days. Cells were subsequently fixed with 2% paraformaldehyde and labelled with DAPI, a dye for nuclear fluorescent staining. Parasitic load were assessed by counting percentages of infected cells in fluorescent microscopy with a magnification of 100 times. Results are shown as representative pictures for control and peracetylated ManNProp treated cells (**a**) or as graphs summarizing three independent experiments as mean±SEM (**b**)

Discussion

The strong dependency on the availability of host's sialic acid to allow *T. cruzi* invasion is well established [9]. Its importance could be elegantly hardened very recently by use of the sialidase inhibitor NeuNAcFNP, which binds covalently to the TS [34]. Here we show that the *N*-acyl side chain of sialic acid of the host cells is the pivotal target to perform this adherence. This finding could be achieved

by biochemical engineering of the *N*-acyl side chain of sialic acid by use of analogues of *N*-acetylmannosamine with prolonged *N*-acyl side chains. Due to the promiscuity of the biosynthetic enzymes novel sialic acids with corresponding modified *N*-acyl side chains are formed [32]. This new biotechnological means is now well established and offers a new tool for glycobiology especially with respect to *N*-acyl modified sialic acids (for review see [20]) and [35]. It is controversially discussed if invasion of mammalian cells by *T. cruzi* depends on TS activity. It is possible to block TS activity by high-density lipoproteins leading to an increased infectivity [36]. On the other hand, Pereira *et al*. (1996) reported the isolation of TS^+ from TS^- trypomastigotes *in vitro* and found significant decreased amounts of parasites attached to the host cell surface and almost no infection using the TS^- parasites whereas the TS^+ trypomastigotes revealed very high infectivity [37]. The Tulahuen strain of *T. cruzi* used for the experiments in this study has also a high expression of TS and exhibit high pathogenicity in mice whereas other strains with low TS exhibit a strongly reduced infectivity in mice [17]. This is in line with our results where desialylation of *T. cruzi* revealed a more pronounced impairment of infection than the treatment of host cells with sialidase C (Fig. 2). Thus, transfer of sialic acids to the cell surface of *T. cruzi* by TS seems to be crucial for a full-blown infection.

The biological importance of the *N*-acyl side chain of the sialic acid is involved in many biological processes, such as development and differentiation of nerve cells [38] or the interaction hemagglutinin of influenza A virus with host's sialic acid; its *N*-acyl modification leads to a 5-fold reduction of infection [12]. Hence, changes in steric characteristics of the sialic acids are responsible for different biological activity of enzymes with sialic acid cleaving activity [11, 39]. However, for *T. cruzi* TS, the catalytic process is not clarified yet but the catalytic site of bacterial and other protozoan sialidases *e.g.* the sialidase from *Trypanosoma rangeli* and also of TS are highly conserved. Our results using a bacterial sialidase showed a pronounced decreased activity towards substrates obtained using ManNBut and ManNPent as sialic acid precursors. These findings are paralleled by a decreased invasion of *T. cruzi* into host cells.

Different domains acting as catalytic sites of bacterial sialidases are described [40–42] and both, the affinity to the carbohydrate binding domain and/or the formation of the catalytic centre [43] could be influenced by a modified acyl side chain. From molecular modelling studies it is evident that the elongation of the *N*-acetyl side chain apparently results in considerable conformational stress on the ligand and in loosening of the interaction that Neu5Ac usually has with the virus surface [11].

From very recent results using *N*-azidomannosamine it is obvious that TS transfers the modified sialic acids to the parasite surface. There is good evidence that the modified sialic acids are mainly transferred to CD45 and CD98 [44]. From the recently described crystal structure of TS the formation of a covalent sialyl-enzyme intermediate is evident with Tyr342 as the catalytic nucleophile [45]. This could be shown by use of 3-fluorosialyl fluoride as a substrate analogue [46].

From the results presented it is concluded that the *N*-acetyl side chain of sialic acid may serve as a target to find potent inhibitors of the invasion of *T. cruzi* into host cells.

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