European Journal of Medicinal Chemistry 132 (2017) 249–261

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Research paper

An *in vitro* and *in vivo* evaluation of new potential *trans*-sialidase inhibitors of *Trypanosoma cruzi* predicted by a computational drug repositioning method

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ARTICLE INFO

Article history:
Received 10 January 2017
Received in revised form
24 March 2017
Accepted 25 March 2017
Available online 28 March 2017

Keywords:
Drug repositioning
Trypanosoma cruzi
Docking
Trans-sialidase
FDA-drugs

ABSTRACT

Chagas disease is one of the most important neglected parasitic diseases afflicting developed and undeveloped countries. There are currently limited options for inexpensive and secure pharmacological treatment. In this study, we employed a structure-based virtual screening protocol for 3180 FDA-approved drugs for repositioning of them as potential *trans*-sialidase inhibitors. *In vitro* and *in vivo* evaluations were performed for the selected drugs against trypomastigotes from the INC-5 and NINOA strains of *T. cruzi*. Also, inhibition of sialylation by the *trans*-sialidase enzyme reaction was evaluated using high-performance anion-exchange chromatography with pulse amperometric detection to confirm the mechanism of action. Results from the computational study showed 38 top drugs with the best binding-energies. Four compounds with antihistaminic, anti-hypertensive, and antibiotic properties showed better trypanocidal effects (LC_{50} range = 4.5–25.8 μg/mL) than the reference drugs, nifurtimox and benznidazole (LC_{50} range = 36.1–46.8 μg/mL) in both strains in the *in vitro* model. The anti-inflammatory, sulfasalazine showed moderate inhibition (37.6%) of sialylation in a *trans*-sialidase enzyme inhibition reaction. Sulfasalazine also showed the best trypanocidal effects in short-term *in vivo* experiments on infected mice. This study suggests for the first time that the anti-inflammatory sulfasalazine could be used as a lead compound to develop new *trans*-sialidase inhibitors.

1. Introduction

Chagas disease (CD) is a neglected parasitic disease caused by the protozoan *Trypanosoma cruzi*. The burden of the disease is calculated to be approximately 7 million infected people dispersed in the United States, Europe [1,2], and Latin America; the latter with the highest prevalence and where 350 million people are at risk for transmission of the parasite [3]. CD (also known as American trypanosomiasis) is the third cause of death by parasitic infections in Latin America only after malaria and esquistosomiasis [4].

Infection in the human host is caused by trypomastigotes deposited in wounds produced by the bite of the triatomine vector. The parasite develops intracellularly until cell disruption, followed by spread of the parasite [5]. During the acute infection phase, individuals are asymptomatic; however, when the disease evolves to the chronic stage, the individual develops digestive and neurological symptomatology or cardiomyopathy that can be potentially fatal [4]. Current medical treatment is based on the use of two drugs, nifurtimox and benznidazole, but they have severe adverse side effects and are not effective in chronic stages of the disease [6]; thus it is necessary to search for new anti-Chagas drugs.

Currently, there are different methodologies to develop and obtain new drugs. A drug repositioning method stands-out among

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http://dx.doi.org/10.1016/j.ejmech.2017.03.063
0223-5234/

these. For instance, the anti-nausea teratogenic drug thalidomide was repositioned for the treatment of leprosy in 1998 and for multiple myeloma in 2006 [7] making it evident that when a new therapeutic property is recognized for a known FDA-approved drug, it can be advanced into clinical trials immediately, saving time and expense [8]. In addition, because the drug-molecules have known safety profiles and effects on organisms, they may be more probably accepted by health authorities, such as the Food and Drug Administration (FDA) of the United States [9].

One way to accelerate the discovery of new therapeutic properties of FDA drugs is through rational structure-based drug discovery. The requisite for this technique is knowledge of a safe druggable target and a crystal protein structure determined experimentally [10]. Some computational studies have been employed for repositioning of FDA drugs as cruzain inhibitors, an essential enzyme of *T. cruzi* metabolism [11–13]. Another well-known protein is *trans*-sialidase (Ts), which plays a relevant role in the host-parasite invasion [14]. Ts is a member of protein sialidases, which are expressed in trypomastigotes, acting through the transfer of the host sialic acid to the mucin of the trypomastigote plasma membrane (the sialylation process), conferring resistance against the complement system of the host [15]. One important fact of Ts is that it lacks human homologous proteins. As a result, Ts protein is a good target candidate for developing new anti-Chagas drugs [16]. This protein has been used as a target to search for new inhibitors using computational drug screening methods [16–18].

In the present research, we performed a computational drug repositioning study using a structure-based virtual screening protocol, and a further *in vitro* and *in vivo* evaluation was performed for repositioning of known FDA-approved drugs against *T. cruzi*. Additionally, an enzymatic assay for Ts was done to confirm the mechanism of action.

2. Results and discussion

2.1. FDA repositioning by computational analysis

The natural compound 3-deoxy-2,3-dehydro-n-acetylneuraminic acid (DANA) included in the 1MS8 pdb file [15] was used as a control in the database. The structure-based virtual screening of 3180 FDA-drugs (available at the time of this writing in ZINC database) predicted 1181 drugs with a better binding energy Vina scores than DANA (-7.7 kcal) (Table S1). From this filtered database 38 top compounds that showed binding-energy scores in a range of -10.0 to -10.9 were selected. Regarding their physicochemical characteristics, most of the molecules complied with Lipinski's rule criteria: molecular weight (MWT) < 500, partition coefficient log P (log P) < 5, number of H-bond donors (H-BD) <5, and number of H-bond acceptors (H-BA) <10 (Table S2). These compounds were then rescored taking into account the z-scores from the Vina score, x-score and drug score measures. The three scores were then used to re-rank the best compounds based on a consensus z-binding score (z-mean) (Table 1), which was applied because it showed better performance over individual scoring functions for prediction of reliable inhibitors in further *in vitro* studies [19]. The 21 best ranked compounds showed the highest MWT >400 (Table S1). Thus, in order to know if MWT influenced the predicted binding-energy, a correlation analysis was performed. The correlation values showed a significant ($p < 0.005$) negative linear relationship between the z-dsx ($r = -0,63$) and z-score ($r = -0,45$) and z-mean ($r = 0,54$) with MWT, indicating that the predicted binding-energies of compounds with a high MWT could be influenced by the molecule size.

In this study, the 38 compounds had a heterogeneous pattern in their chemical structure, reflecting their different FDA indications (Table 1). For instance, included among these compounds were antibiotics, antihypertensives, antidiabetics, antipsychotics, antihistamines, anti-inflammatory, antineoplastics, anticoagulants, steroids and a few other drugs without available information about their commercial or biological use.

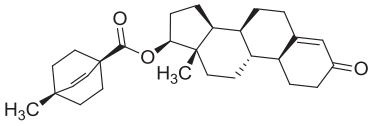
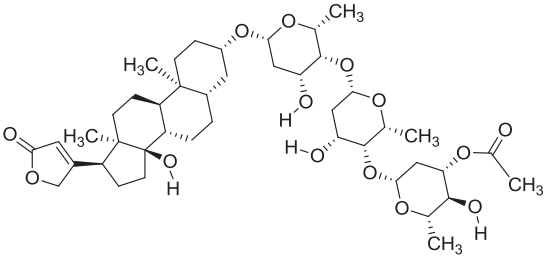
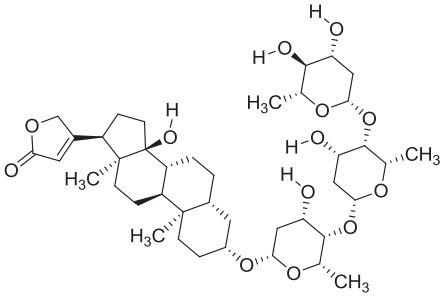
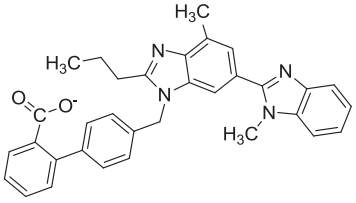
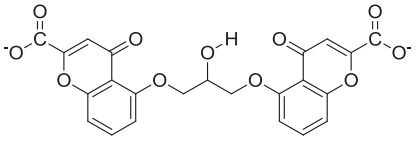
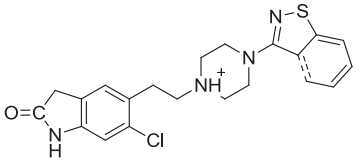
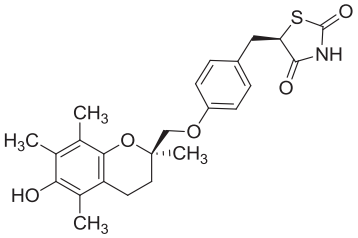
To understand their chemical heterogeneity and to help us choose drugs with known FDA information as well as their purchase availability, a clustering analysis was performed based on the ligand contact with the amino acids on the active site. The Ts active site architecture contains essential amino acids such as the arginine triad (Arg35, Arg245, and Arg314), involved in the binding of carboxylate groups present in all sialic acid derivatives, a glutamic acid (Glu357) that stabilizes Arg35, and an aspartic acid (Asp59) essential for catalysis; furthermore, there are two residues (Tyr342 and Glu230) in the floor that participate in the stabilization of the transition state of the reaction [15]. Thus our analysis was focused on the contact of the compounds with those essential amino acids. The matrix of ligand amino acid contact (Supplemental material 2) generated by AuPosSOM software showed that the 38 compounds interact with most of the essential catalytic residues mentioned above (except Glu357). For example, the best ranked drug ZINC03831187 (13-methyl-3-oxo-2,6,7,8,9,10, 11, 12,14,15,16,17-dodecahydro-1H-cyclopenta [a]phenanthren-17-yl) interacts through hydrophobic bonds with essential amino acids (Asp 59, Arg 245, Tyr 342, and Glu230) on the Ts active site (Fig. 1).

This matrix was used to draw a cluster tree that grouped the compounds according to the most similar amino acid contact. The tree shows six major groups and 12 leaves (Fig. 2), but most of the drugs were clustered in the 1 (n = 7), 3 (n = 6), 4 (n = 10) and 6 (n = 8) groups. Group 3 contains the best ranked compound ZINC03831187 (z-mean = −1.6232593567), characterized by dodecahydro-1H-cyclopenta phenanthren as the main chemical structure. In the same group, the compound ZINC03830430 appeared with low scoring (z-mean = 0.1512051127). This compound is characterized by a β-lactam ring and is known as the antibiotic cefoperazone. Hence, based on the previous observation, it was decided to test drugs within the four major groups which had a reported FDA indication and commercial availability for purchase, despite the lower z-mean score compared with the best ranked compounds within the group. Thus, ZINC03830430 (z-mean = 0.151) and ZINC03830847 (z-mean = 1.512) were taken from group 3, ZINC03812892 (z-mean = −0.839), ZINC00537877 (z-mean = 0.105), and ZINC00896512 (z-mean = 0.824) from group 4, and ZINC03830467 (z-mean = −0.911) and ZINC03831490 (z-mean = 1.05) from group 6.

2.2. Anti Trypanosoma cruzi activity

From the computational study data, seven compounds were chosen for screening at a concentration of 50 μg/mL to determine their potential trypanocidal effects (% lysis) on blood samples infected with trypomastigotes from the NINOA and INC-5 strains (Table 2). The drugs tested showed different trypanocidal effects. In the initial lysis assay, four drugs showed trypanocidal effects in the range of 75–100% for the INC-5 strain (Cefsulodine, sulfasalazine and flubendazole showed a lysis effect <50%), and six drugs had trypanocidal activity in the range of 64–100% for the NINOA strain, only flubendazole showed a value < 50%. The same biological behavior was also observed in the lysis concentration media (LC_{50}) evaluation. Only four drugs showed better anti-parasitic effects in both strains than the reference drugs. The antihypertensive doxazosyn mesilate (LC_{50} = 12.91 ± 1.4 μg/mL) and the antihistamine terfenadine (LC_{50} = 4.5 ± 0.6 μg/mL) showed the highest LC_{50} values on NINOA and INC-5 strain, respectively. The antibiotic cefsulodin and the anti-inflammatory sulfasalazine, showed good

Table 1
The chemical structure of the best ranked FDA drugs and their indication.

ZINC ID	Chemical Structure	Z-mean	FDA Indication[a]
03831187		−1.623	Analogue cardiotonic steroid
08101051		−1.453	Analogue cardiac glycoside
08101078		−1.219	Analogue cardiac glycoside
01530886		−1.106	Antihypertensive (Telmisartan)
01530788		0.047	Mast cell stabilizer (Cromolyn sodium)
00538550		0.09	Antipsychotic (Ziprasidone hydrochloride)
00968272		0.101	Antidiabetic and anti-inflammatory (Troglitazone)

(*continued on next page*)

Table 1 (*continued*)

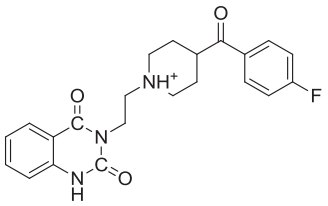
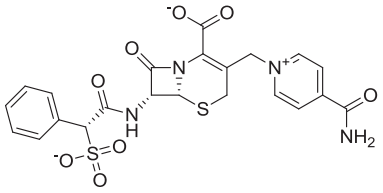
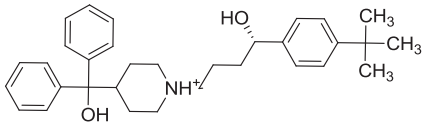
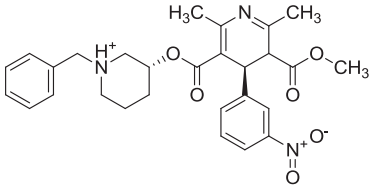
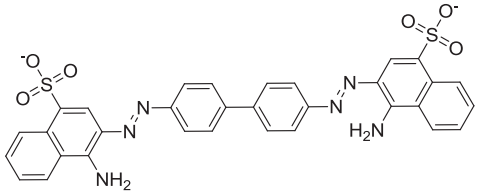
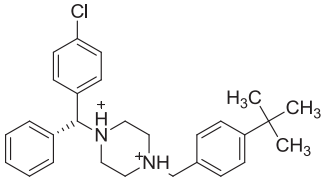
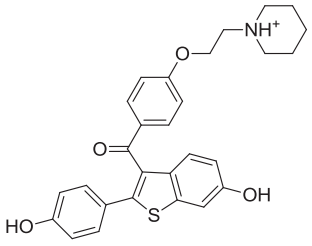
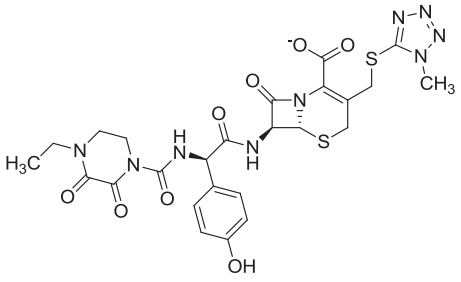
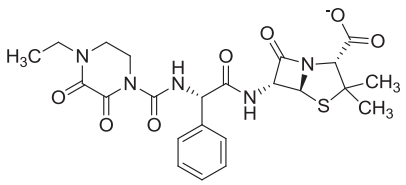
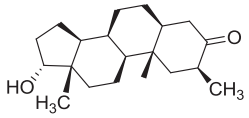
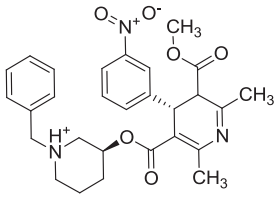
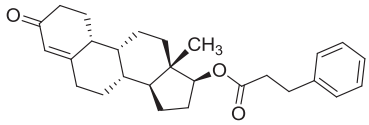
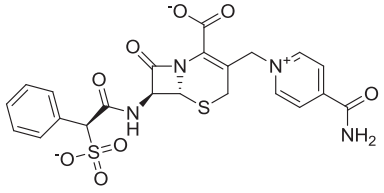
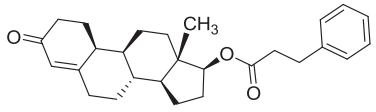
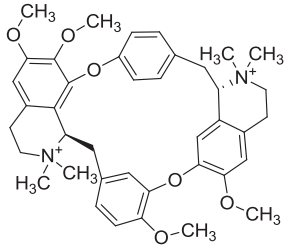
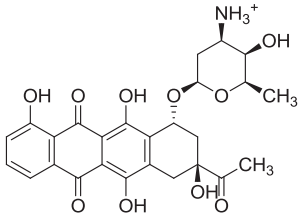
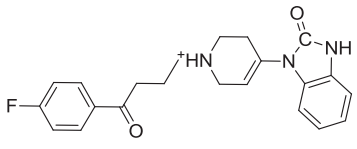
ZINC ID	Chemical Structure	Z-mean	FDA Indication[a]
00537877		0.105	Antihypertensive (Ketanserin)
03830467		−0.911	Antibiotic (β-lactam, Cefsulodin sodium)
03812892		−0.839	Antihistamine (Terfenadine)
03800980		−0.740	NAI
03830554		−0.501	NAI
01530909		−0.473	NAI
00538275		0.106	Selective estrogen modulator (Raloxifene hydrochloride)
03830430		0.151	Antibiotic (β-lactam) (Cefoperazone)

Table 1 (*continued*)

ZINC ID	Chemical Structure	Z-mean	FDA Indication[a]
11592733		0.204	Analogue Azlocillin β-lactam
08552458		0.332	Analogue to anabolic steroid, hormonin
03830271		0.373	NAI
04026871		−0.461	Antipsychotic (Ziprasidone hydrochloride)
01542915		−0.424	Antidiabetic and anti-inflammatory (Troglitazone)
03881612		−0.387	Antihypertensive (Ketanserin)
04198846		−0.306	Selective estrogen modulator (Raloxifene hydrochloride)
03830383		0.419	Antracycline antibiotic
00601282		0.498	NAI

(*continued on next page*)

Table 1 (*continued*)

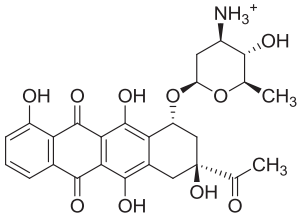
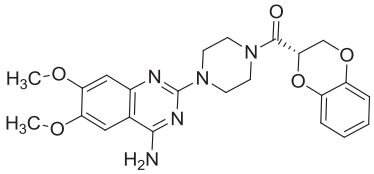
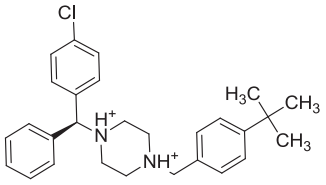
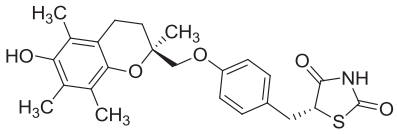
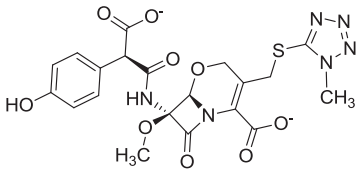
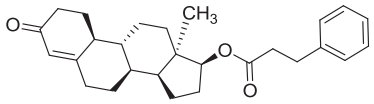
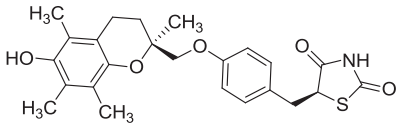
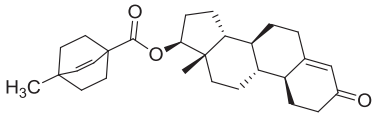
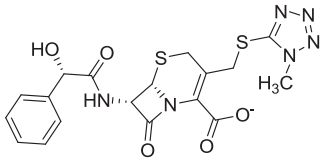
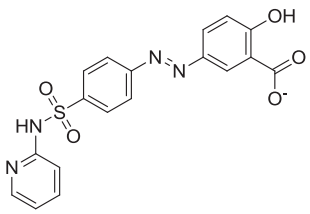
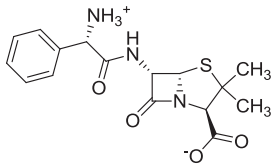
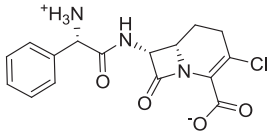
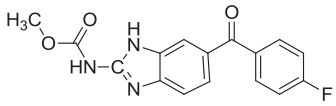
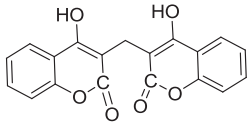
ZINC ID	Chemical Structure	Z-mean	FDA Indication[a]
03830385		0.764	Antracycline antibiotic
00896512		0.824	Antihypertensive (Doxazosin Mesylate)
01530908		−0.300	Antibiotic (β-lactam, Cefoperazone)
00968279		−0.188	Analogue Azlocillin β-lactam
03831159		−0.130	Analogue to anabolic steroid, hormonin
03831190		−0.101	NAI
00968277		−0.035	Antracycline antibiotic
3831189		0.002	NAI
01530586		0.881	Analogue to Cefonicid β-lactam
03831490		1.05	Anti-inflammatory (Sulfasalazine)

Table 1 (*continued*)

ZINC ID	Chemical Structure	Z-mean	FDA Indication[a]
04523361		1.059	Analogue β-lactam
01851195		1.065	Analogue β-lactam
03830847		1.512	Anthelmintic (Flubendazole)
00020258		1.592	Anticoagulant (Dicoumarol)

NAI= Not available information about their FDA indication in ZINC database Chemspider, PubChem, and ChEMBL.
[a] FDA indication based on ZINC database and search query on Chemspider, Google, PubChem, and ChEMBL.

trypanocidal effects only on the NINOA strain. For the drugs with a percentage of lysis <50% at first-concentration lower than the reference drugs in both strains the LC_{50} was not determined.

2.3. Enzyme inhibition studies

The inhibitory properties of six selected drugs towards Ts from *T. cruzi* (TcTS) were measured in their capacity to inhibit sialylation of *N*-acetyllactosamine in the reaction exemplified in Fig. 3. Thus, 3'-sialyllactose (1 mM) as a donor, *N*-acetyllactosamine (1 mM) as an acceptor, and TcTS were incubated in the absence or presence of inhibitor (1 mM) and the incubation mixtures were analyzed by high performance anion exchange chromatography with pulse amperometric detection (HPAEC-PAD) [21].

Inhibition was determined by comparing the amounts of 3'-sialyl-*N*-acetyllactosamine obtained in the presence or absence of the tested compound. When equimolar concentrations of donor, substrate and inhibitor were used, inhibition values ranged between 0 and 37% (Fig. 3). Sulfasalazine was the only compound that showed moderate inhibition (37.6%) and its analysis by HPAEC is shown as an example (Fig. 4). Therefore, these results suggest that the other tested drugs could have trypanocidal activity through a different mechanism of action.

According to the docking analysis, sulfasalazine is interacting with most of the essential amino acids of the active site of Ts (Fig. 5) as follows: sulfapyridine moiety is binding through a hydrophilic interaction with two amino acids of the arginine triad (Arg35, and Arg314) and with hydrophobic bonds with the catalytic Tyr342 amino acid, hydroxyl group of the mesalazine moiety is interacting with the catalytic Asp59 and with two other amino acids (Arg93, Trp120) through hydrophilic bonds; moreover, we also observed that azo bond of sulfapyridine-mesalazine complex it is interacting with Tyr119 amino acid. This amino acid is involved in the conformational switch of sialic-acid for the activation of Ts enzyme [22]. Thus, based on these observations, we suggest that the chemical modifications on the mesalazine moiety could help to obtain better Ts inhibitors.

2.4. Short-term in vivo studies

In order to know the potential trypanocidal effect in humans, a short-term *in vivo* assessment was performed with four drugs on NINOA-infected mice (Fig. 6A): the antihistamine terfenadine, the anti-inflammatory sulfasalazine and the antibiotics cefoperazone and cefsulodin. Terfenadine and cefoperazone were only tested in mice infected with the INC-5 strain (Fig. 6B). In mice infected with the INC-5 strain (Fig. 6B), treatment with benznidazole showed a decrease of parasitemia at all times measured. In terfenadine and cefoperazone treatment, a decrease of parasitemia was observed at 2 and 4 h; however, at 6 h, parasitemia increased. This could be due to a decrease of drug concentration in the circulatory system. In the NINOA strain, the reference drug benznidazole also produced a decrease of parasitemia in all the tested times (Fig. 6A). Mice treated with these four drugs showed a decrease of parasitemia at 2 and 4 h; however, at 6 h (final time), parasitemia remained unchanged or had a small increase, but for sulfasalazine treatment, a decrease of parasitemia of nearly 60% was observed at 4 h. This is interesting because this compound showed better *N*-sialylation inhibition effects on the Ts inhibition study. Therefore, of all the drugs in the test set, the anti-inflammatory sulfasalazine showed the highest trypanocidal effect in short-term *in vivo* experiments on infected mice. Sulfasalazine has been successfully used for the treatment of inflammatory cardiomyopathy in ankylosing spondylitis [23,24]. Thus, the anti-inflammatory effect that sulfasalazine possesses might be beneficial for the inflammatory cardiomyopathy produced by the parasite in late stages of the disease, in addition to the possible Ts inhibition. These results encourage future sulfasalazine chemical modifications along with long-term *in vivo* evaluations, more carefully considering additional pharmacokinetic parameters such as absorption, distribution, and the general effects of its metabolism.

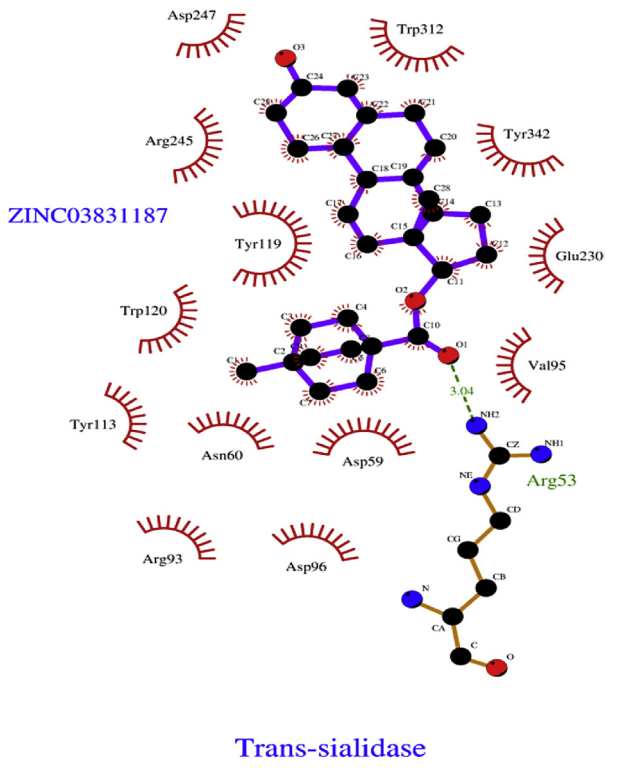


Fig. 1. The best ranked compound, ZINC0383118, docked on the Ts active site. Arcs with red lines represent amino acid hydrophobic contacts; green dashed lines represent hydrogen bonds. The 2D image was produced with LigPlot software [20]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

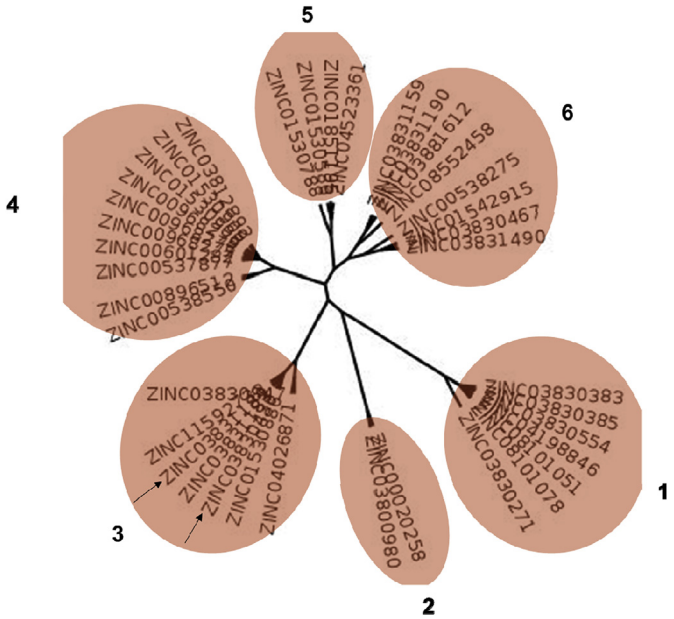


Fig. 2. Grouping patterns of the 38 top ranked FDA-drugs. The image was prepared using the Figtree software (http://tree.bio.ed.ac.uk/software/figtree/).

3. Conclusion

In this work, a computational screening protocol was applied to evaluate FDA-drugs as potential TcTS inhibitors. The *in vitro* evaluation carried out on trypomastigotes showed that four FDA-drugs have trypanocidal effects in both INC-5 and NINOA strains. These effects were maintained during the *in vivo* short-time assays but with lower efficacy in comparison with the reference drug benznidazol. The enzyme inhibition studies using HPAEC-PAD demonstrated that sulfasalazine is a moderate Ts inhibitor (37.6%). The antiparasitic effect of this compound was the most pronounced during the *in vivo* studies. Thus, the compound sulfasalazine could be used as a starting point to develop new TcTS inhibitors with anti-inflammatory properties which could be beneficial to treat the infection and complications of CD.

4. Materials and methods

4.1. Database creation and docking protocol

The structure-based virtual screening was carried out on 3180 (Supplementary material 1) FDA-approved drugs retrieved from the ZINC database website [25]. These 3180 compounds were used to create a dataset of compounds using the prepare_ligand4.py python script from AutoDock Tools [26]. The script allows merging nonpolar hydrogens, adding Gasteiger charges, and setting up rotatable bonds for each ligand to produce the pdbqt file format to be used as input for the AutoDock Vina software [27]. The latter was employed for the docking process. The Ts (PDBID 1MS8) crystal structure [15] was retrieved from the Protein Data Bank (PDB), and was used as a receptor removing the ligand 3-deoxy-2,3-didehydro-*N*-acetylneuraminic acid (DANA) and water molecules, for the docking process. The AutoDock Tools software was used to prepare the receptor molecule and add the polar hydrogens and Gasteiger charges; finally, the Vina configuration files were generated.

The first docking process was carried out with DANA (ligand control) in order to determine the size of search spaces on the active site for Ts. DANA was placed in the active site and several rounds of dockings were carried out to increase the size of search space. The final size space dimension for Ts was x = 16, y = 16, and z = 16 Å, and the center 40.881, 63.047, and -37.451 for x, y and z, respectively. The binding energy obtained for the control (DANA) was introduced into the ZINC dataset to be used as the cutoff value to select potential inhibitors based on the Vina scoring function which takes into account the sum of all atom pairs; i.e., the intermolecular and intramolecular contributions. The compounds with high binding-energy values above the cutoff were considered inactive compounds; therefore, the high negative top-binding energy compounds were considered potential inhibitors. From a preliminary set of inhibitors, the best ranked potential inhibitors were chosen to perform a consensus score (z-mean) using the scoring functions Vina [27], DrugScore [28] and X-score [29]. The X-Score and DrugScores programs computes their respective scoring function based on the three-dimensional structure of the protein-ligand complexes. These two functions are not included in a specific docking program, thus the docking coordinates obtained form AutoDock-Vina was used as input for both programs. The z-mean based on the average of z-score for each dataset formed by the three scoring functions was calculated as described Liu et al., [30]. In brief, Z-scores were calculated considering the scoring value obtained for each compound within each scoring group (Vina, DrugsScore, and Xscore), minus the mean value calculated from each scoring group, and divided by the standard deviation. The Z-scores obtained for each compound within the scoring groups were used to re-rank the compounds based on the Z-mean (consensus score). Furthermore, we also analyzed the physicochemical features of the best ranked compounds, and the possible influence of the molecular weight was assessed thorough a Pearson correlation performed with the software R [31].

Table 2
Percentage of lysis and lysis concentration media of the selected FDA drugs on *T. cruzi* strains.

Name	Clinical use	% lysis on INC-5 at 50 μg/mL	LC_{50} (μg/mL) on INC-5 strain	% lysis on NINOA at 50 μg/mL	LC_{50} (μg/mL) on NINOA strain
Terfenadine	Antihistamine	100	4.5 ± 0.6	100	16.3 ± 0.6
Doxazosin mesylate	Antihypertensive	89	14.7 ± 1.2	97	12.91 ± 1.4
Ketanserin tartrato	Antihypertensive	73	18.4 ± 2.1	89	13.6 ± 1.5
Cefsulodin sodium	Antibiotic	14	ND	87	16.6 ± 0.9
Cefoperazone sodium	Antibiotic	71	23 ± 1.8	64	25.8 ± 0.7
Sulfasalazine	Anti-inflammatory	10	ND	92	19.3 ± 1.2
Flubendazol	Antihelmintic	42	ND	41	ND
Benznidazol	Antichagasic	56	40.6 ± 2.4	69	46.6 ± 1.9
Nifurtimox	Antichagasic	51	46.7 ± 5.2	66	33.1 ± 1.3

ND= Not determined.

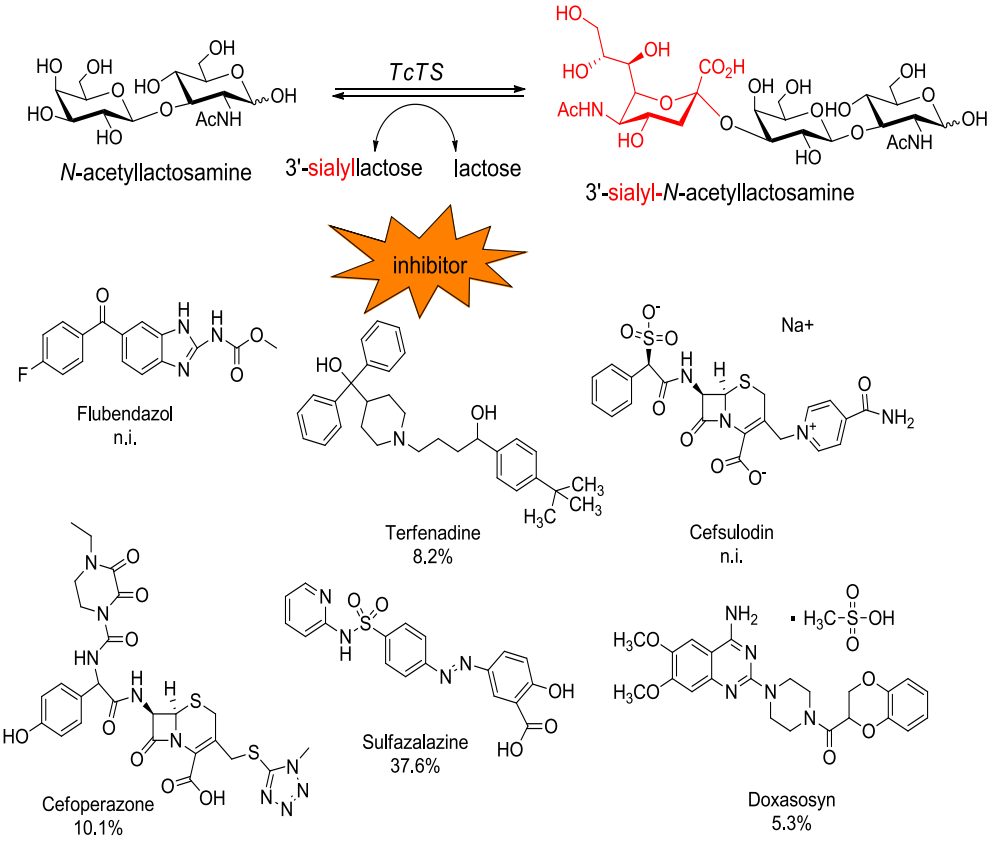


Fig. 3. Sialylation of *N*-acetyllactosamine catalyzed by TcTS as a target of the inhibitors tested. The inhibition percentages are indicated in each structure; n.i.: no inhibition.

4.2. Clustering and ligand-amino acid contact analysis

Ligand-contact amino acid analysis was performed for the top ranked compounds obtained from the docking study. AuPosSOM (Automatics analysis of poses using SOM) [29] software was employed for the clustering process which involved three steps, 1) first a Kohonen self-organizing map (SOM) is trained using ligand-amino acid descriptors, 2) then an unsupervised cluster analysis is performed, 3) finally a Newick tree file is generated for the visual analysis of compounds grouped according to similar active site binding.

4.3. In vitro trypanocidal assays

Seven drugs were purchased from the Sigma Aldrich company (Sigma names and codes: sulfasalazine-S0883, cefsulodin-C8145, terfenadine-T9652, cefoperazone-C4292, doxazosin mesylate-D9815, flubendazol-34091, and ketanserin tartrate-S006) for the *in vitro* evaluations. The *in vitro* trypanocidal assays were carried out according to the protocols reported elsewhere, with slight modifications [32–38]. Blood infected with trypomastigotes from *T. cruzi* INC-5 and NINOA strains were obtained by cardiac puncture from infected NIH mice at the peak of infection and adjusted to 1×10^6 forms/mL. Stock solutions (10 mg/mL) in DMSO of each test compound and reference drugs were prepared and subsequent dilutions were done with sterile distilled water. The seven compounds were dissolved in dimethyl sulfoxide (DMSO) to a concentration of 50–100 μg/mL. The final concentration of DMSO in the culture medium remained below 1%. A solution of DMSO/H_2O (1:99) was used as a negative control. The test was carried out three times on 96-well microplates (Biofil JET) containing 195 μL of infected blood and 5 μL of the compounds per well. At the start of

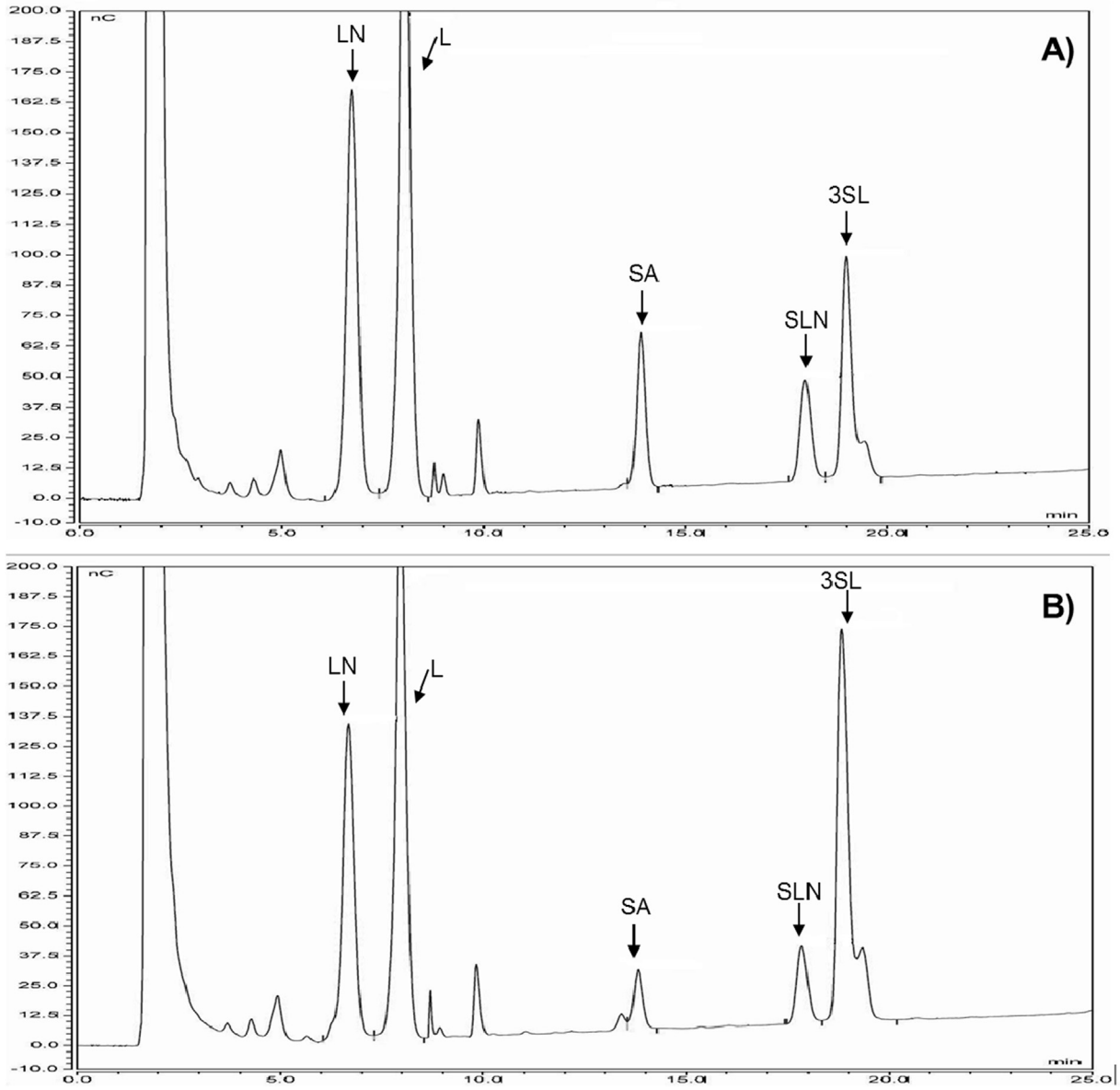


Fig. 4. HPAEC-PAD graphical analysis for *N*-acetyllactosamine, 3'-sialyllactose and TcTS incubated in the absence (4A) or presence of (4B) sulfazalasine. L: lactose; SA: sialic acid; LN: *N*-acetyllactosamine; 3SL: 3'-sialyllactose.

the *in vitro* experiments, the trypanocidal activity of the seven compounds was assessed at 50 μg/mL. Then, the compounds with a lysis percentage >50 were proved at five concentrations (100–10 μg/mL) to get the lysis concentration of 50% of the population (LC_{50}). LC_{50} values were determined using Probit statistical analysis of the dose-response, and the results were expressed as mean ± standard deviation (SD). As negative control of lysis, wells with untreated blood trypomastigotes were used, and as positive control, wells with reference drug were used. The plates were incubated for 24 h at 4 °C to avoid the change to the epimastigote phase [33]. Bloodstream trypomastigotes were counted by the Brener method [39]. Briefly, 5 μL of blood were spread on slides, covered with a coverslip, and flagellates were examined with an optical microscope at 40× magnification. Anti-*T. cruzi* activity was expressed as lysis percentage by comparing the remaining trypomastigotes in each concentration with respect to the negative control group. Each assay was performed three times for each *T. cruzi* strain.

4.4. Inhibition of sialylation of N-acetyllactosamine (LN)

Reaction mixtures of 20 μL containing 20 mM Tris-HCl pH 7 buffer, 30 mM NaCl, 1 mM 3'-sialyllactose as donor, 1 mM *N*-acetyllactosamine and 1 mM of the compounds cefsulodin, terfenadine, cefoperazone, doxazosin mesylate, flubendazol, and ketanserin tartrate were incubated with 300 ng of purified Ts from *T. cruzi* [34] or 15 min at room temperature. Samples were then diluted 12 times with deionized water and analyzed by high-performance anion-exchange chromatography with pulse amperometric detection (HPAEC-PAD). Inhibition was calculated from the amount of 3'-sialyl-*N*-acetyllactosamine with respect to the total amount of sialylated compounds obtained in the presence or absence of the tested compound. Since flubendazol, sulfazalazine, doxasosyn and terfenadine insoluble in water, they were dissolved in DMSO (10 mM) and 2 μL of these solutions were used in the incubations. The control assay, in the absence of the inhibitor, was performed accordingly, adding 2 μL of DMSO to the incubation

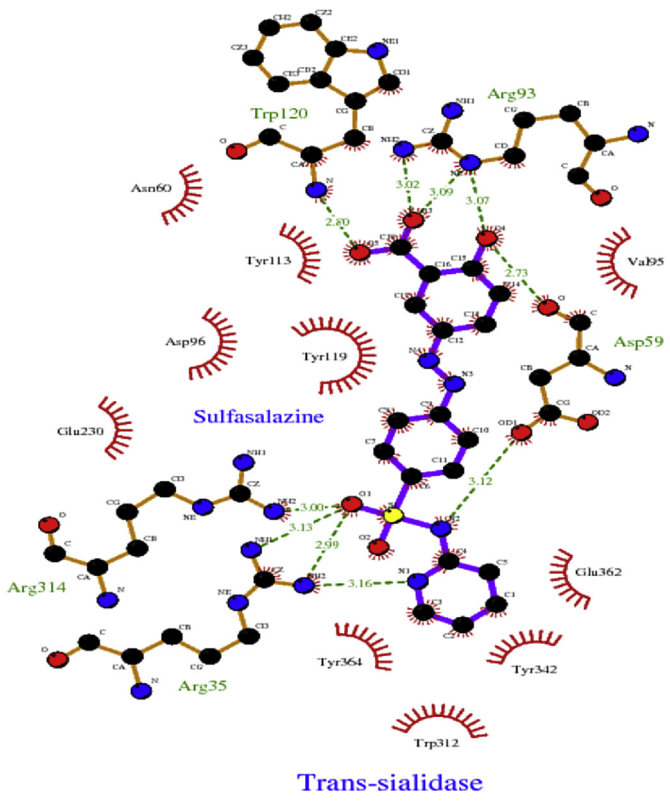


Fig. 5. Sulfasalazine docked on the *trans*-sialidase active site. Arcs with red lines represent amino acid hydrophobic contacts; green dashed lines represent hydrogen bonds. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

mixture [40,41].

4.5. Analysis by HPAEC-PAD

HPAEC-PAD was performed using a Dionex ICS 5000 HPLC system with a pulsed amperometric detector and a CarboPac PA-100 ion exchange analytical column (4 × 250 mm) equipped with a PA-100 guard column (4 × 50 mm). Elution was performed in 80 mM NaOH with a linear gradient from 0 to 500 mM NaAcO in 60 min at a flow rate of 0.9 mL/min.

4.6. In vivo evaluation against T. cruzi

Short-term *in vivo* assessments were performed emulating the Filardi-Brener [42], and Romanha technique [43]. Briefly, assemblies of five NIH female mice (20–25 g) were inoculated intraperitoneally with 2×10^5 blood circulating trypomastigotes from the INC-5 and NINOA strains. Four FDA-approved drugs (cefsulodin, cefoperazone, terfenadine and sulfasalazine), and the reference drug benznidazole, were suspended in 4% gum arabic (Sigma Aldrich G9752). During the peak of parasitemia (19th-24th days), compounds were administered orally to mice to be tested for a total dose of 100 mg/kg. The controls were administered only with the vehicle. Parasitemia was measured at 2, 4 and 6 h after compound ingestion utilizing blood from the tail. The rate in percentage for diminished parasitemia was assessed microscopically contrasting the number of blood trypomastigotes acquired at every time interval. Mice examinations were performed as stated in the Norma Official Mexicana (NOM-062-Z00-1999) published on August 22, 2009.

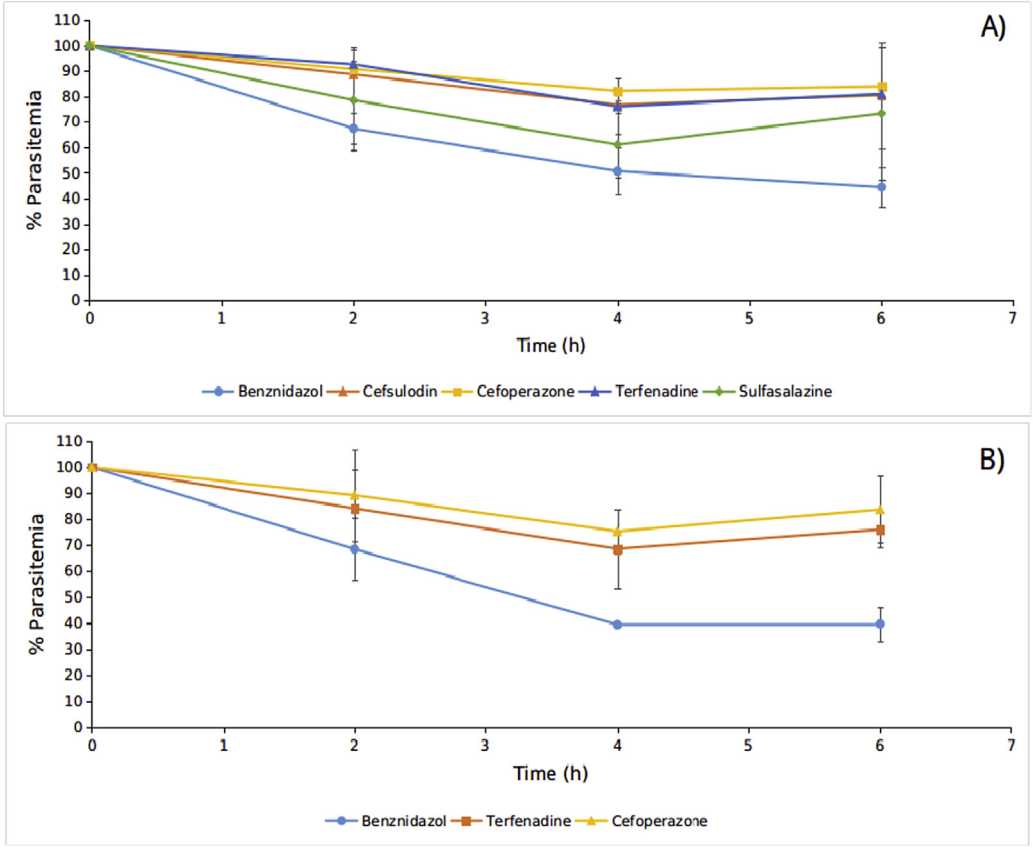


Fig. 6. Short-term *in vivo* evaluation of FDA-drugs selected on NINOA (A) and INC-5 (B) strains.

Acknowledgments

We wish to express our gratitude to the Consejo Nacional de Ciencia y Tecnología (Proyecto Apoyado por el Fondo Sectorial de Investigación para la Educación, CB-2014-01, 241615) for their financial support. Gildardo Rivera and Benjamín Nogueda-Torres hold a scholarship from the "Comisión de Operación y Fomento de Actividades Académicas" (COFAA-IPN) and the "Programa de Estímulos al Desempeño de los Investigadores" (EDI-IPN). We thank Oscar Campetella and his group from the Universidad Nacional General San Martín (UNSAM) Argentina, for their kind gift of *trans*-sialidase from *T. cruzi*. Support for this work from the Universidad de Buenos Aires, the CONICET and the ANPCyT is gratefully acknowledged. RA and MLU are research members of the CONICET.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2017.03.063.

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