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Original article

Computer-guided drug repurposing: Identification of trypanocidal activity of clofazimine, benidipine and saquinavir

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A B S T R A C T

In spite of remarkable advances in the knowledge on *Trypanosoma cruzi* biology, no medications to treat Chagas disease have been approved in the last 40 years and almost 8 million people remain infected. Since the public sector and non-profit organizations play a significant role in the research efforts on Chagas disease, it is important to implement research strategies that promote translation of basic research into the clinical practice. Recent international public-private initiatives address the potential of drug repositioning (i.e. finding second or further medical uses for known-medications) which can substantially improve the success at clinical trials and the innovation in the pharmaceutical field.

In this work, we present the computer-aided identification of approved drugs clofazimine, benidipine and saquinavir as potential trypanocidal compounds and test their effects at biochemical as much as cellular level on different parasite stages. According to the obtained results, we discuss biopharmaceutical, toxicological and physiopathological criteria applied to decide to move clofazimine and benidipine into preclinical phase, in an acute model of infection. The article illustrates the potential of computer-guided drug repositioning to integrate and optimize drug discovery and preclinical development; it also proposes rational rules to select which among repositioned candidates should advance to investigational drug status and offers a new insight on clofazimine and benidipine as candidate treatments for Chagas disease.

One Sentence Summary: We present the computer-guided drug repositioning of three approved drugs as potential new treatments for Chagas disease, integrating computer-aided drug screening and biochemical, cellular and preclinical tests.

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1. Introduction

Drug repositioning (i.e. finding new therapeutic uses for already known drugs including marketed, discontinued and shelved drugs, and yet-to-be-pursued clinical candidates) has gained increasing attention within the international drug development community over the last few years [1–5]. Repositioned drugs represent unique translational opportunities, including substantially higher probability of success to market than new drugs, and a reduced development timeline to potentially 3–12 years [6,7]. Repurposed candidates have survived preclinical toxicological testing and proved tolerable safety and adequate pharmacokinetic profiles. When the repurposed drug has previously been used in clinical

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practice, manufacturing and stability issues have already been solved; furthermore, many drugs are off patent and may provide relatively inexpensive solutions as therapies for other diseases [8]. Successful drug repurposing stories have probably contributed to this interest; e.g. sildenafil was originally investigated for the treatment of hypertension and ischemic heart disease but has acquired blockbuster status as a treatment for erectile dysfunction. Aspirin itself has expanded its therapeutic indications and is now widely used to prevent heart attacks and strokes in patients with existing cardiovascular disease.

Second uses have been majorly found through serendipitous observations (e.g., intelligent exploitation of unforeseen side-effects). Lately, however, rational knowledge-based repositioning strategies have been explored, including chemoinformatic-, bioinformatic- and network-based approaches [9–15] and high-throughput literature analysis [16,17]. Repositioning has been signaled as a particularly useful strategy for the discovery of new treatments for rare and neglected diseases [18–20]. These disorders frequently offer limited potential revenue to pharmaceutical companies and are addressed by private-public joint efforts, the academic sector or non-profit organizations.

Here, we present the application of computer-aided drug discovery in the search of novel treatments for Chagas disease. Chagas disease is a tropical parasitic disease caused by the flagellate protozoan *Trypanosoma cruzi* [21]. Although a series of control campaigns developed by World Health Organization (WHO), Pan American Health Organization (PAHO) and national authorities have considerably reduced Chagas disease incidence in the last fifteen years, there are still almost 8 million infected people and 28 million people at risk in Latin America [22,23]. The human disease occurs in two stages: an acute stage, which occurs shortly after an initial infection, and a chronic stage that develops thereafter. The acute phase lasts for the first few weeks or months of the infection. It usually occurs unnoticed because it is symptom-free or exhibits mild, unspecific symptoms. The lifelong chronic stage frequently remains asymptomatic; however, around 30% of the patients will develop clinical affections on the heart, the digestive system or the nervous system. About two-thirds of people with chronic symptoms present cardiac damage, including dilated cardiomyopathy which causes heart rhythm abnormalities and may result in sudden death. About one-third of patients go on to develop digestive system damage, resulting in dilation of the digestive tract (megacolon and megaesophagus), accompanied by severe weight loss [24,25]. Current treatment against Chagas relies only on two approved drugs developed during 1960s-1970s, namely nifurtimox and benznidazole, which seem to be ineffective in the late chronic phase of the disease and present severe side-effects and resistance issues [26,27]. Important advances, however, have been made in the fields of biochemistry and molecular biology of *T. cruzi* and potential novel therapeutics [23,28–30]. Cruzipain (Cz), the major cystein protease of the parasite, has particularly been explored as new drug target [31–33], proving to be essential for replication of the intracellular form of *T. cruzi* and playing a role in host-parasite interactions [32–36]. Our group has previously applied computer-guided drug repositioning for the search of novel Cz inhibitors [37,38]. In those reports, Dragon software was used for molecular descriptor calculation. Although those models were able to identify dose-dependent Cz inhibitors, the effective concentrations were much higher than those used for the original indication, discouraging further investigation. Here, we have used DESMOL software for the computation of molecular descriptors included in the model, hoping to identify better candidates for repositioning. An integrative approach is presented for the search of novel anti-chagasic agents, including virtual screening (VS) of Cz inhibitors oriented to rational drug repositioning and subsequent biochemical, cellular and pre-clinical testing. A set of biopharmaceutical, toxicological and physiopathological criteria have been applied to decide which of the tested candidates would progress to the pre-clinical stage.

2. Materials and methods

2.1. Dataset compilation and splitting

A 147-compound balanced dataset including 77 Cz reversible inhibitors and 70 non-inhibitors was compiled from literature [39–50]. In order to split the dataset into representative training and test sets, the LibraryMCS v0.7 (ChemAxon) hierarchical clustering approach was applied in combination with the k-means clustering implemented in Statistica 10 Cluster Analysis module (Statsoft Inc, 2011). LibraryMCS relies on similarity guided Maximum Common Substructure (MCS, i. e. the largest subgraph shared by two chemical graphs) to cluster a set of chemical structures without exhaustive pairwise comparison.

2.2. Descriptor calculation and modeling

A set of well-known topological descriptors was used in this work: Subgraph Randic-Kiel-Hall like indices up to the fourth order (${}^{m}X_{t}$, ${}^{m}X_{t}^{v}$) [51,52] topological charge indices, TCI, up to the fifth order, (J_m, G_m, J_m^v, G_m^v) [53], quotients and differences between valence and non-valance connectivity indices (${}^{m}C_{t} = {}^{m}X_{t}/{}^{m}X_{t}^{v}$ and ${}^{m}D_{t} = {}^{m}X_{t}\text{-}{}^{m}X_{t}^{v}$), PRn (number of pairs of ramifications at topological distance n, with n ranging from 0 to 4), Vn (number of vertices with topological valence n, with n being 3 or 4). Each compound was characterized by a set of 62 descriptors. All descriptors used in this work were obtained with the aid of the DESMOL11 software [54].

Linear Discriminant Analysis (LDA) was then conducted in order to derive a classification model capable of distinguishing Cz inhibitors from non-inhibitors. LDA is a qualitative supervised learning method aimed to finding a linear combination of independent variables to differentiate between two or more categories of objects. Each object class is associated to a given value (an integer value) of an arbitrary variable that serves as class label. In our case, only two object classes (ACTIVE – Cz inhibitors and INACTIVE – non-inhibitors) were considered, thus the class label assumes two observed values (1 and −1, respectively). Since the output of the function being searched is not a continuous variable but only an object category, LDA and other classificatory techniques may be useful to handle noisy data, e.g. if a given experimental endpoint is associated to large variability or if experimental data from a diversity of laboratories are compiled [55].

The Discriminant Analysis module of Statistica 10 was used to build the models. A tolerance value of 0.5 was selected in order to exclude highly correlated descriptors from the model. All the coefficients linked to the models descriptors were significant at a 0.05 level. A minimum ratio of 15 between the number of training set compounds and the number of independent variables was used in order to reduce the chance of overfitting. Parsimony principle, Wilks' lambda and the performance of the model on the independent test set were used to select the best model. Standard validation approaches (stratified leave-group out cross-validation, randomization test and external validation) were used to assess the model's robustness and predictive ability [56]. Stratified 12-fold cross-validation and 15 randomization tests were applied.

2.3. Virtual screening

VS refers to the application of computational models or algorithms as filters to select drug candidates from chemical

repositories or databases. Here, we have conducted VS on Merck Index 12th database. The Merck Index contains over 5570 chemical compounds, drugs or groups of related compounds. Receiver Operating Characteristic (ROC) curves were constructed for both the training and test sets, in order to select the discriminant function score threshold value determining an adequate sensitivity/specificity ratio, to be used in the VS campaign [57,58]. To built ROC curves MedCalc ROC curves analysis tool was applied (Medcalc software, 2012).

2.4. Docking studies

Saquinavir, benidipine, clofazimine and the inactive compound verapamil were docked and scored by Autodock 4.2 docking software [59]. The starting protein was prepared from the crystal structure of one reversible inhibitor complexed to cruzain (PDB code 1ME4, resolution limit 1.1 A) [60]. The compounds were docked employing the Lamarckian genetic algorithm in the "docking active site", defined through a 19 × 15 × 15 A^3 grid, centered on the relative position of the experimental ligand in the crystallographic structure. We employed the default grid spacing (0.375 Å) and performed 100 docking runs for each compound, treating the docking active site as a rigid molecule and the ligands as flexible, i.e., all non-ring torsions were considered active. We also set the default Autodock parameters for all the variables such as Marsilli–Gasteiger partial charges.

2.5. Inhibitory effect on Cz activity assay

To study the effect of the selected compounds on Cz activity, the enzyme was partially purified by ammonium sulfate precipitation followed by affinity column chromatography on concavalin A-sepharosa (Sigma), as previously described [61]. The activity of the partially purified Cz was assayed with 250 μM Bz-Pro-Phe-Arg-pNA (Sigma) as substrate, incubated in a buffer of 6, 5 μM dithiothreitol (DTT) and 50 mM Tris–HCl pH 7 [62], in presence or absence of the tested candidates. The reaction was measured spectrophotometrically at room temperature at 410 nm for 5 min (Beckman CoulterTM DU530 Life Science UV–vis spectrophotometer.). The values obtained were converted into pmol of hydrolyzed substrate per min by using the extinction coefficient 8.800 M^{-1} cm^{-1} (p-nitroanilides). The inhibitory effect of the selected candidates was expressed as the percentage of residual activity of Cz respect to the assay without inhibitors.

2.6. Inhibitory effects on *T. cruzi* epimastigote proliferation

Epimastigotes of the *T. cruzi* strain Y were cultured at 28 °C in BHT medium with 20 mg/L Haemin, 20% heat-inactivated fetal calf serum and antibiotics (100 μg/mL streptomycin and 100 U/mL penicillin) [63] adding clofazimine, benidipine or saquinavir at the indicated concentration (0–100 uM). Cultures were initiated at 10^7 cells/mL and the proliferation was followed daily by cell counting in a hemocytometer chamber.

2.7. Trypomastigote invasion and amastigote proliferation assays

Epimastigotes of *T. cruzi* Y strain that express GFP -kindly provided by Dr S. Schenkman, Departamento de Microbiologia, Imunologia e Parasitologia, Universidade Federal de Sao Paulo, (Sao Paulo S.P., Brazil)- were differentiated to metacyclic trypomastigotes in TAU and TAU-AAG media and used to infect Vero cells according to described previously [64], Tissue cell trypomastigotes (TCT) obtained from the infected Vero cells were maintained in cell culture and prepared as described previously [65]. H9C2 rat myoblasts cells (ATCC CRL-1446) were maintained in D-MEM (Gibco Laboratories, Bs. As. Argentina) supplemented with 10% FBS (# 16,000-044, Gibco) and antibiotics at 37 °C in an atmosphere of 95% air and 5% CO2. For experiments, cells were plated on coverslips in 24 well plates. Tissue cell trypomastigotes (TCT) were treated with drugs for 30 min before infection and then transferred to H9C2 cell monolayers for an infection period of 12 h in D-MEM supplemented with 3% SFB and antibiotics (Infection medium) in absence or presence of different concentration of Cz inhibitors. Unbound trypomastigotes were removed by washing with PBS. Infected monolayers were incubated by an additional period of 48 h in Infection medium supplemented with the compounds to study the effect of Cz inhibitors in the first stages of infection. Amastigote proliferation assays were conducted in a similar way but using different times. H9C2 cells were infected for a period of 24 h without inhibitors and treated during 96 h in the presence of Cz inhibitors before fixation. Cells were then washed 3 times with PBS, fixed with 3% paraformaldehyde solution in PBS for 15 min at room temperature, and quenched with 50 mM NH_4Cl. Subsequently, cells were permeabilized with 1% saponin in PBS containing 1% bovine serum albumin (BSA), and incubated with TRITC-phalloidine (Sigma, St. Louis, MO, USA) to stain cellular limits. Cells were mounted with Mowiol containing the DNA marker Hoechst (Life Technologies, Bs. As., Argentina) and examined by confocal microscopy as previously described [64]. Percentage of infected cells and number of amastigotes per cell were quantified for each condition and analyzed with the test of multiple comparisons of Tukey–Kramer using the KyPlot programme.

2.8. Criteria to move to animal models

Three basic criteria were used to prioritize candidates testing in an acute mice model of infection. Briefly, we analyzed: a) whether the effective concentrations on *T. cruzi* cells were similar to those steady state plasma concentrations attained during a multi-dosage regime for the previously approved therapeutic indication of the repositioned candidate. Ideally, equal or lower doses to those used for the original therapeutic indication should be employed for our pursued indication (trypanocidal effect); b) whether the previous indications might pose any additional benefit to the patient with Chagas disease (e.g. an antichagasic agent with additional cardioprotective effect might help to control not only the infection but also symptoms of the disease) and; c) the severity of the known adverse effects associated to the doses used for the original indication. Ideally, the repurposed candidate should have less frequent and/or mild side-effects compared to the already available medications (i.e. nifurtimox and benznidazole). Moreover, candidates with certain side-effects incompatible with Chagas symptoms (e.g. drugs contraindicated in cardiac patients) should be excluded as potential Chagas treatments.

2.9. Animal infection

Mice of C57BL/6J strain, including both sexes, ranging from 7 to 9 g of initial weight [66], were used in an acute model of infection. The animals were maintained in specific pathogen-free conditions (SPF) and housed in temperature-controlled rooms (22–25 °C) and received water and food *ad libitum*. All animal procedures were performed in accordance with bioethic rules of the *Comité Institucional para el Cuidado y Uso de Animales de Laboratorio* (*CICUAL*), *Facultad de Ciencias Médicas, Universidad Nacional de Cuyo*.

The animals were divided into four experimental groups of 3 animals each:

Group 1, control: infected animals treated with saline solution.

Group 2: infected animals treated with Benidipine (10 mg/kg/day).
Group 3: infected animals treated with Clofazimine (20 mg/kg/day).
Group 4: infected animals treated with Benznidazol (100 mg/kg/day).

Animals were inoculated by intraperitoneal route with 100,000 tissue cell trypomastigotes (TCT) forms of *T. cruzi* Y-GFP strain. At 5 days post infection (5 dpi) parasitemia is performed and treatment is initiated in animals showing circulating forms of *T. cruzi* and continued until the day of death. Treatment was administrated daily in a volume of 200 μl into the intraperitoneal cavity. All treatments were prepared in 1% DMSO. Parasitemia was evaluated by counting the parasite number present in peripheral blood taken from the tail vein by direct puncture. At 5 dpi was performed by microscopic examination of a drop of blood (5 μl) under coverslip and immediately examined and quantified the parasite number in 50 fields (400×) [66]. At 10, 14 and 20 dpi, parasitemia was assessed by counting the parasites in a Neubauer chamber and the results were expressed in parasite number/ml. Survival rates were determined by daily inspection of the cages.

3. Results

3.1. Modeling and virtual screening

The following topological model capable of differentiating Cz inhibitors from non-inhibitors was obtained through LDA:

$$\text{Class} = -4.762 + 1.325 * \text{G4V} - 1.248 * \text{X4P} + 0.421 * \text{G1} + 0.171 * \text{VV3}$$

$$N = 105 \; F(4,100) = 33.44 \; \lambda = 0.428 \; p < 0.00001$$

where G4V = Valence topological charge index of order 4; X4P = connectivity index of order 4 type path; G1 = Topological charge index of order 1; VV3 = number of vertices with valence 3. The model presents an excellent observations/predictor ratio (around 25) which indicates a low chance of overfitting. When using 0 as a score threshold to differentiate active from inactive compounds, the model presents 86% of good classifications among the training set inactive compounds, 91.7% of good classifications among the training set active compounds, and an overall of 88.5% good classifications. Regarding the independent test set, the model accurately classifies 81% of the active and 85.7% of the inactive compounds, with an overall good classification of 83%. Note that the performance on the test set is very similar to the performance on the training set, confirming no overfitting. The average performance of the randomized models was 65.7% (sd = 4.6) showing that the randomized models were significantly outperformed by the real model, as expected. The 12-fold cross-validation resulted in an average percentage of good-classifications of 85.8%, which is remarkably similar to the external validation results.

We resorted to Receiving Operating Characteristic (ROC) curves in order to optimize the chosen threshold score on a rational basis, balancing the model's sensitivity (Se) and specificity (Sp). The area under the curve (AUC) for the training and test sets ROC curves were, respectively, 0.931 and 0.862 (1 represents an ideal performance, while 0.5 represents random selection). On the basis of the results, a score of 0.5 was defined as the cut-off value to differentiate active from inactive compounds in the VS campaign. According to the ROC curves data, this corresponds to a sensitivity of 77% and a specificity of 98% in the training set and a Se of 71% and a Sp of 95% in the test set. In the selection of the cut-off value, we have prioritized Sp over Se to avoid the selection of false positives in the screening campaign.

The model was applied in the VS of 5570 small molecules from Merck Index 12th Edition. 154 candidates belonging to the model's applicability domain presented a model score above the selected threshold; 34 of them correspond to approved drugs, which are the most favorable, direct candidates for repositioning purposes. On the basis of their accessibility, four of them (Fig. 1) were acquired and experimentally tested in enzymatic assay on Cz crude extract. The acquired candidates were verapamil (used in the treatment of hypertension and cardiac arrhythmia), clofazimine (an anti-leprosy agent with anti-inflammatory actvity), benidipine (a channel blocker used in the treatment of hypertension and ischaemic heart disease) and saquinavir (an antiretroviral drug used in HIV therapy).

3.2. Enzymatic testing

As a first biochemical approach, the effect of the four selected candidates was tested at 100 μM on Cz activity from *T. cruzi*, using Bz-Pro-Phe-Arg-pNA as chromogenic substrate. Bibliographic search and the NCBI Pubchem Bioassay Database analysis revealed that clofazimine has previously been tested on Cz with ambiguous results. Doak et al. reported inhibitory activity in both presence and absence of Triton 0.01% (reported IC50s of 40 and 6 μM), while a quantitative high-throughput screening study by Jadhav et al. which assessed the effect of more than 197,000 candidates on the hydrolysis of the fluorogenic substrate Z-Phe-Arg-AMC by Cz in absence and presence of detergent reported no inhibitory activity on Cz (AIDs: 1476, 484274) [67,68]. Thus, we decided that it was worth rechecking clofazimine inhibitory activity and, if positive, proceed to study its effect on *T. cruzi* proliferation and infectivity. Jadhav et al. were inconclusive regarding the saquinavir inhibitory effect on Cz, while we found no previous reports on verapamil or benidipine activity.

While verapamil did not affect Cz activity, the three other compounds showed significant inhibitory effects (Fig. 2). Such inhibitions showed to be dose-dependent (data not shown).

3.3. Docking studies

To analyze the possible mode of action of the inhibitors on Cz, we simulated the interaction between the most active compounds (saquinavir, benidipine, and clofazimine) and the enzyme by computational docking. We employed Autodock 4.2 software with the conditions described in *Materials and methods* section. These conditions were selected based on their capacity to reproduce the orientation of the co-crystallized inhibitor in the experimental complex (ligand encoded as T10 in 1ME4 [60]). Fig. 3 shows the superimposition of the top score conformation obtained from the docking and the experimental structure. Additionally, the binding energies predicted by docking evidence an acceptable correlation with the biological data, since the scores obtained for saquinavir, benidipine, and coldazimine were −12.76 kcal/mol, -8.42 kcal/mol and −7.36 kcal/mol respectively whereas the score for inactive compound verapamiil was −6.37 kcal/mol.

We evaluated the orientation predicted by docking for the molecules relative to the cruzain active site (Fig. 4). The best ranked pose of clofazimine shows interactions with the S2 pocket of the active site through one of its chlorophenil groups. This lipophilic region was defined by the residues Leu67, Ala133 and Leu157 and it is susceptible to interact with aromatic groups as shown in the experimental complex 1ME4 [60]. The other chlorophenil group of clofazimine avoids interactions with the enzyme and it is exposed to the solvent. Similarly, docking simulations for saquinavir predict interactions between its benzyl ring and the S2 pocket, which is reinforced by the decahydro-isoquinoline ring placed into the S3

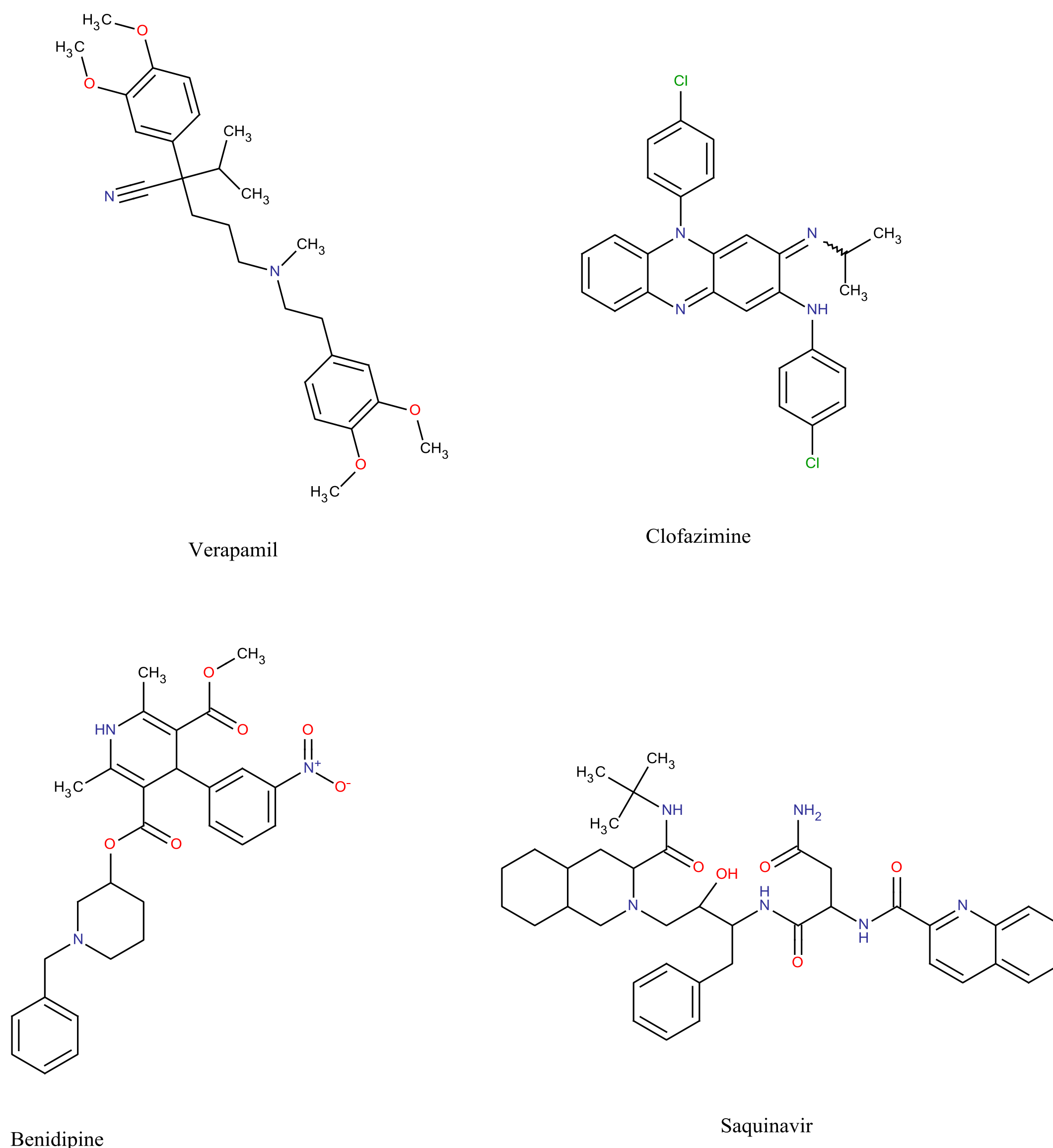

Fig. 1. Molecular structures of the four candidates selected for enzymatic testing.

region. Regarding benidipine, its best ranked conformation is oriented in a way that stabilizes interactions between the nitrophenyl ring and the S3 region, and it exposes its methyl-ester group to the solvent. No strong interactions were detected between the inhibitors and the catalytic triad (Cys25, His159 and Asn175), so we propose that the stability of the complexes was ruled by the interactions with the regions near the catalytic cleft previously mentioned. However, more molecular simulations will be performed in the future to support our hypothesis.

3.4. Biological studies

The ability of clofazimine, benidipine and saquinavir to affect *T. cruzi* proliferation was next tested in biological assays on different *T. cruzi* parasitic forms.

Initially, the effect of the compounds was tested on growth curves of epimastigotes (the proliferative and non-infective stage, present in the insect vector), showing that the candidate drugs affect *T. cruzi* epimastigotes proliferation in a time and dose-dependent manner (Fig. 5) presenting values of median lethal concentration (LC50) around 10.6 μM for clofazimine (Fig. 5A) and 19.5 μM for benidipine (Fig. 5B) at the middle log phase of controls (4th day). Higher concentrations than 20 μM of clofazimine resulted lethal in few hours. Saquinavir also affected epimastigotes proliferation (Fig. 5C), however its effect was seen tardily, at stationary phase; higher concentrations were not tested because of saquinavir low solubility in DMSO. The three compounds also showed a notorious effect on epimastigote morphology and motility as soon as the first, second or third day of culture, depending on the drug and in its concentration (data not shown).

Remarkably, the median effect on epimastigotes were obtained at lower concentrations than the effective ones observed in the enzymatic assay, which would suggest that the candidates are targeting other molecular targets besides Cz.

Since it is known that Cz plays a role in cell invasion, Cz inhibitors were also studied on trypomastigotes (the infective host form of *T. cruzi*) by using a method previously described [69]. Tissue culture trypomastigotes (TCT) were pre-treated for 30 min with the inhibitors and exposed to myoblast monolayers for 12 h. Unbound trypomastigotes were removed by washing and infected

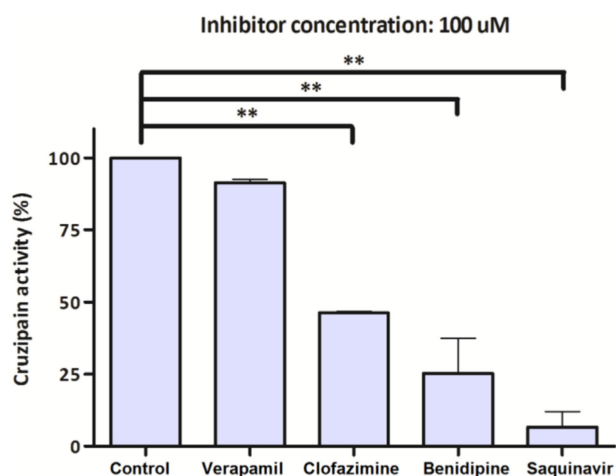

Fig. 2. Inhibitory effect of the four selected candidates on purified Cz activity from *T. cruzi*. The final concentration of each compound was 100 μM. Protease activity is expressed as percentage of the control condition (2% DMSO). Results represent the mean of two independent experiments. Asterisks indicate significant differences compared to the control (**$p < 0.01$).

monolayers were incubated in the presence of drugs during 48 h for parasite multiplication (see scheme in Fig. 6A and details in *Materials and Methods*). Each inhibitor was assayed at a no cytotoxic concentration, as defined by other authors (2.5–10 μM) [70–72]; conveniently, these concentrations were in the LC50 range determined in the epimastigotes assays (shown in Fig. 5). Infection was quantified by confocal microscopy of fixed samples. Parasites were directly visualised due to the stable expression of Tc H2b fussed to GFP [73] and host cells were observed by TRITC-phalloidin staining of actin cytoskeleton (Fig. 6B).

As shown in Fig. 6C, the three candidates significantly reduced cell invasion by more than 3 folds, measured as percentage of host cells infected. Complementary, number of parasites in each cell was also significantly reduced indicating that early division of recently

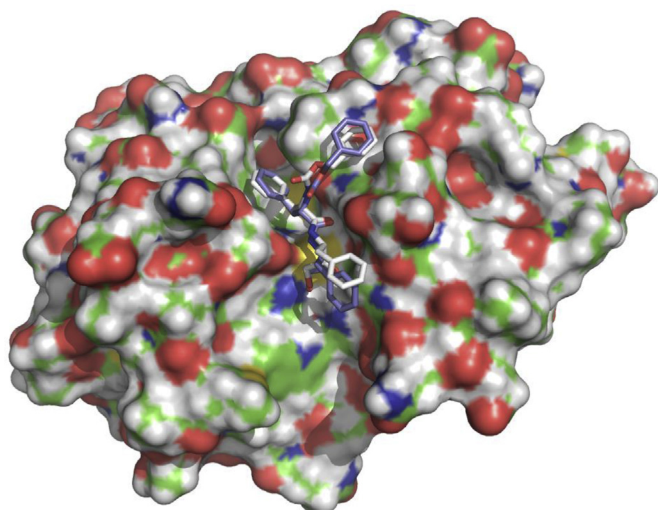

Fig. 3. Superposition of the docking conformation (carbon atoms in white) and the crystallographic ligand (carbon atoms in violet) in the active site. Hydrogen atoms of the inhibitors were omitted for simplicity. Color code for the protein as follows: nitrogen atoms in blue, hydrogen atoms in white, oxygen atoms in red and carbon atoms in green. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

formed amastigotes is impaired in the presence of the tested drugs (Fig. 6D).

After that, we analyzed the effect of Cz inhibitors on amastigote proliferation. In this case, cells were infected during 24 h in control medium and, after being washed, they were exposed to drugs and incubated by an additional time of 96 h (Fig. 7A). Interestingly, quantitative data showed that Cz inhibitors also reduced the number of amastigote per cell (Fig. 7B).

Despite each inhibitor was assayed at a no cytotoxic concentration, we have also explored the metabolic activity of host cells treated with Cz inhibitors by using the Alamar blue assay as previously shown [65]. As expected treated cells display similar vitality than controls (data not shown). Importantly, the candidate drugs have selective action over *T. cruzi* at assayed concentrations.

3.5. Candidate selection to move to preclinical stage

Clofazimine is mainly indicated in the treatment of leprosy [74]. Doses of 200–300 mg daily are administered under medical supervision, for no more than 3 months, for the treatment of erythema nodosum leprosum. It is worth highlighting that current medications for Chagas disease are also administered in two/three-month length treatments. Although the absorption of orally administered clofazimine is variable, daily p.o administration of 100, 300, 400 and 600 mg of clofazimine to leprosy patients results in average plasma levels of 1.5, 2.1, 3.0 and 8.5 μmol, respectively [74]; in other words, the steady state levels of clofazimine achieved with the doses used for the previous indication are quite similar to the effective concentrations observed on *T. cruzi* assays. The adverse effects of clofazimine are generally dose-related, primarily affecting the skin, eyes and gastrointestinal tract. They are tolerable and gradually reversible on cessation of therapy. The most common gastrointestinal tract side effects are mild to moderate (abdominal/epigastric pain, nausea, diarrhea, vomiting); less frequently, however, gastrointestinal effects might be severe, requiring medical supervision for high doses. The coincidence between the clofazimine levels that show antichagasic effect and the ones used for the previously approved clinical indication, plus the reasonable side-effects profile made clofazimine a good candidate for further investigation.

Benidipine is a calcium channel blocking agent currently used for the treatment of hypertension and angina pectoris [75]. Interestingly, abundant evidence indicates that benidipine has cardioprotective properties [76–79], which might represent an additional benefit for patients with Chagas. Its more frequent side-effects are relatively mild and related to their vasodilative activity, including palpitations, hot flushes and headache. Furthermore, many other clinically used dihydropiridines have previously shown antitrypanosomal effects [80].

Saquinavir was not moved to the mice model because, although generally well tolerated (the more frequent adverse effects are mild gastrointestinal symptoms) the mean maximum steady state concentration is around 0.15 μM [81], way below the concentrations that affected epimastigote proliferation, trypomastigote invasion and amastigote proliferation. Moreover, being an antiviral there is no evidence or suggestions that it might present any additional benefit to the patient with Chagas.

3.6. In vivo analysis in acute infection mice

The compounds selected to move to the animal model, clofazimine and benidipine, were individually tested in an experimental acute murine infection model at doses of 20 mg/kg and 10 mg/kg respectively (Fig. 8). 1% DMSO (negative control) or 100 mg/kg Benznidazole (positive control) were administered in two other

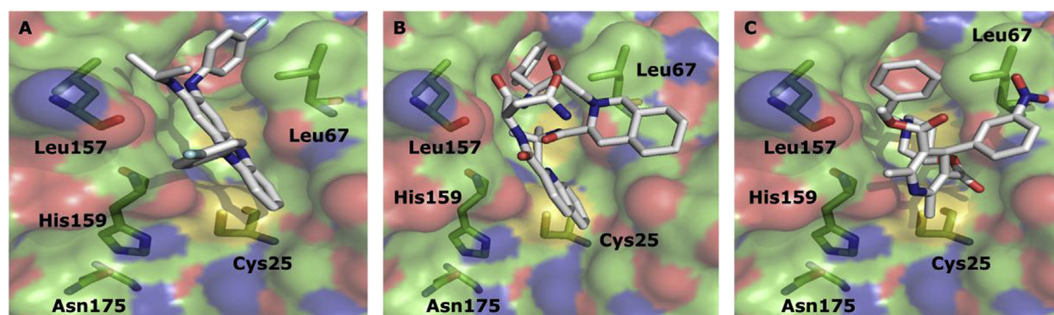

Fig. 4. Surface representation of the cruzain active site in complex with clofazimine (A), saquinavir (B) and benidipine (C). Representative residues are highlighted in green. Color code as follows: carbon (protein) in green, carbon (inhibitors) in white, nitrogen atoms in blue, oxygen atoms in red. Hydrogen atoms were omitted for simplicity. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

animal sets to include untreated and reference drug groups respectively. High parasitemia values were displayed by untreated animals at day 14 post infection (14 dpi) while Benznidazole group displayed the minor value at these conditions. Benidipine and clofazimine significantly reduced parasitemia around 40–60% compared to control values (Fig. 8A).

We have also conducted a post-mortem analysis of heart parasitism by conventional microscopy of cardiac tissue sections (See details in *Materials and Methods*). HE staining permits the clear observation of *T. cruzi* nests between muscular fibers (Fig. 8B). Our data show that number of cardiac amastigote nests was significantly reduced in the hearts of clofazimine treated animals; benznidazole also produced a low cardiac parasitism, as expected (Fig. 8C). Taken together, these results highlight the beneficial action of clofazimine treatment on the animal infection process and justify further experiments to a) optimize the administered dose in acute infection tests and; b) move forward to chronic models of mice infection.

4. Discussion

We have developed a computational model capable of detecting Cz inhibitors with high predictivity. The cut-off output score of the model has been optimized with the help of ROC curves to increase the model's Sp and minimize the number of false positives (compounds predicted as Cz inhibitors but that do not possess inhibitory activity).

The reported model was applied in a VS campaign on Merck Index 12th, finding four candidates. The inhibitory effect was confirmed in Cz enzymatic assays and in epimastigote cultures. As three of them were clearly Cz inhibitors (though unknown additional action mechanisms are possibly present), these compounds were tested in *in vitro* infection and intracellular proliferation assays. Different susceptibilities of the parasite to inhibitors were observed through the assays from purified Cz and epimastigotes to more complex models involving tripomastigotes and amastigotes within the host cells. Cz is a complex mixture of isoforms, most of

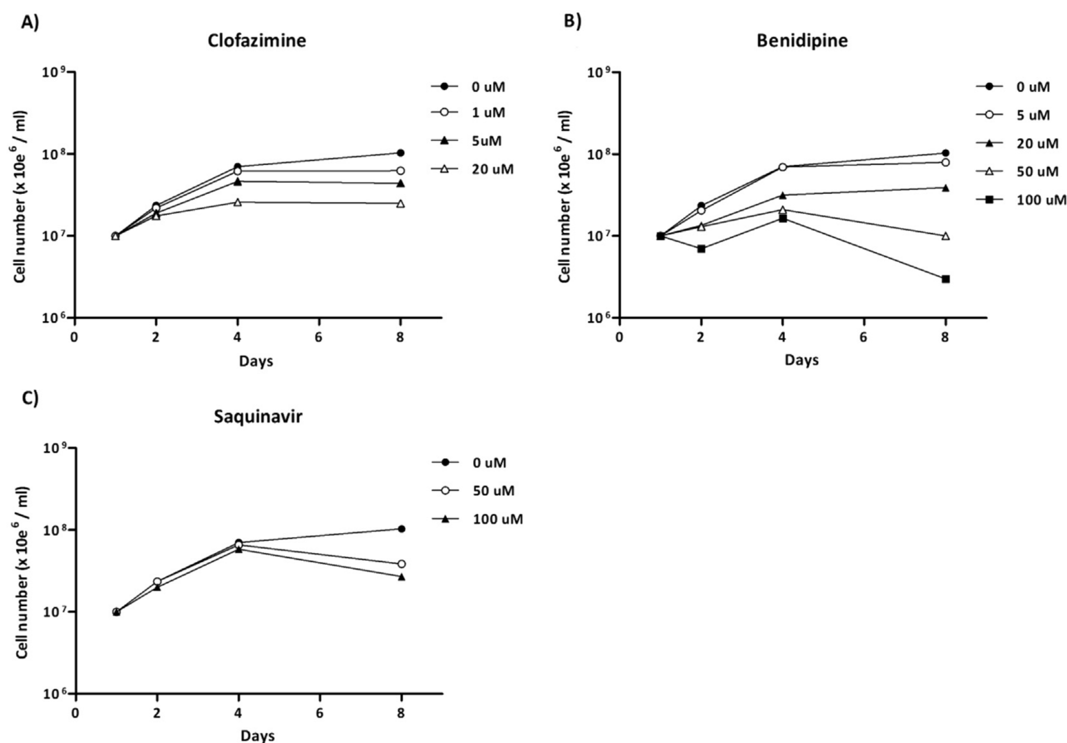

Fig. 5. Effects of clofazimine (A), benidipine (B) and saquinavir (C) on proliferation of *T. cruzi* epimastigotes. To determine the growth rate at each condition, 10^7 cells/ml were seeded in BHT medium and maintained at 28 °C for eight days. The range of concentration tested in each case depended on their solubility; the control condition was done with 2% DMSO. Parasites were counted periodically using a hemocytometer chamber. One of three independent experiments with similar results is shown.

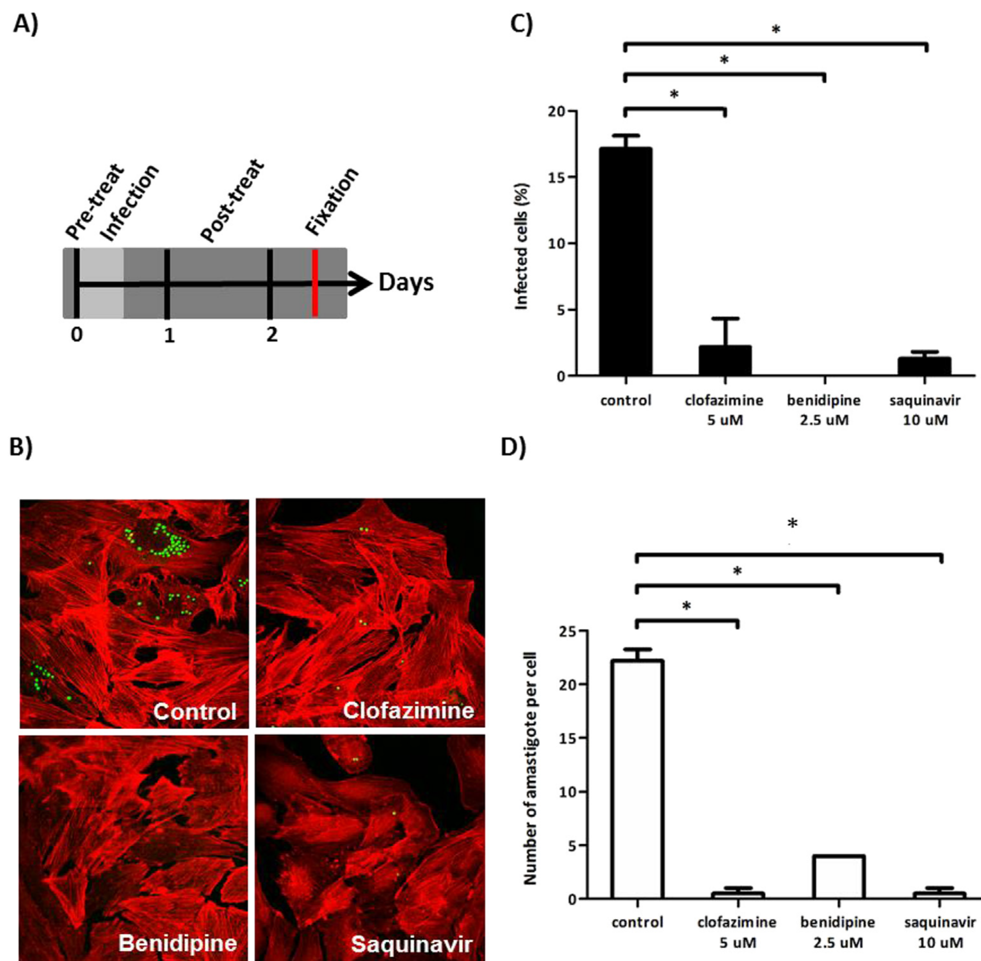

Fig. 6. Effects of clofazimine, benidipine and saquinavir on *T. cruzi* infectivity. TCT from *T. cruzi* Y-GFP strain were pre-treated with Cz inhibitors for 30 min and then were applied to H9C2 cells monolayers (MOI = 10) during an invasion period of 12 h. Cells were next washed and fresh medium -with or without inhibitors-was added and incubated for an additional period of 48 h. Cells were subsequently fixed, stained and prepared for microscopic analysis as described in *Material and Methods*. Images were obtained in an Olympus FV1000 confocal microscope and Tukey–Kramer test were used for statistical analysis of data. A: Schematic representation of the performed protocol. B: Confocal images depicting cells from each treatment: *T. cruzi* amastigotes are visualized in green and cellular actin in red. C: Percentage of infected host cells. D: number of parasites per cell at the indicated inhibitors. Data represent the mean ± SE of at least three independent experiments (number of counted cells ≈100). Asterisks indicate significant differences from controls (*p < 0.05). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

them are grouped in the cruzipain 1 family, but there are other isoform, termed cruzipain 2, which are expressed preferentially in the mammalian stages of *T. cruzi* and differs markedly in pH stability, substrate specificity and sensitivity to inhibition [82]. The existence of these isoforms differentially expressed in the parasitic forms could explain our observations.

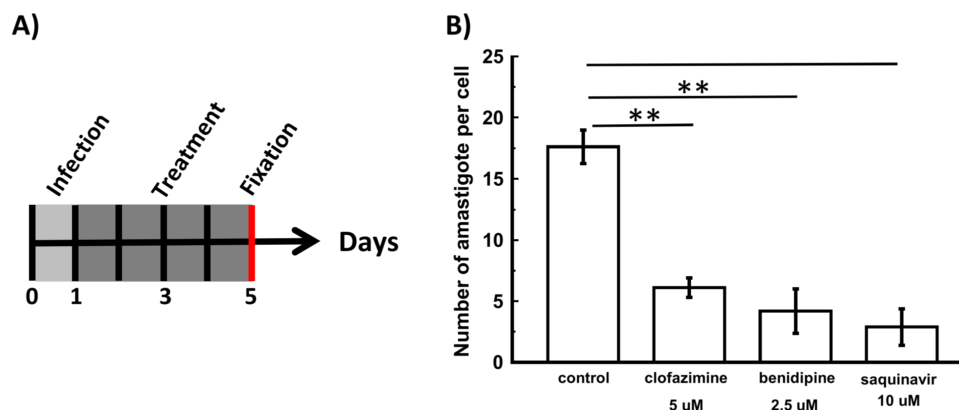

Fig. 7. Effects of clofazimine, benidipine and saquinavir on proliferation of *T. cruzi* amastigotes: TCT from *T. cruzi* Y-GFP strain were used to infect H9C2 cells monolayers (MOI = 10) during 24 h. Cells were next washed and fresh medium with inhibitors was added and incubated for an additional period of 96 h. Cells were subsequently fixed, stained and prepared for microscopic analysis as described in Material and Methods. IA: Schematic representation of the performed protocol. B: number of amastigote per cell at the indicated inhibitors. Data represent the mean ± SE of at least three independent experiments (number of counted cells ≈ 100). Asterisks indicate significant differences from controls (** p < 0.01; *** p < 0.001).

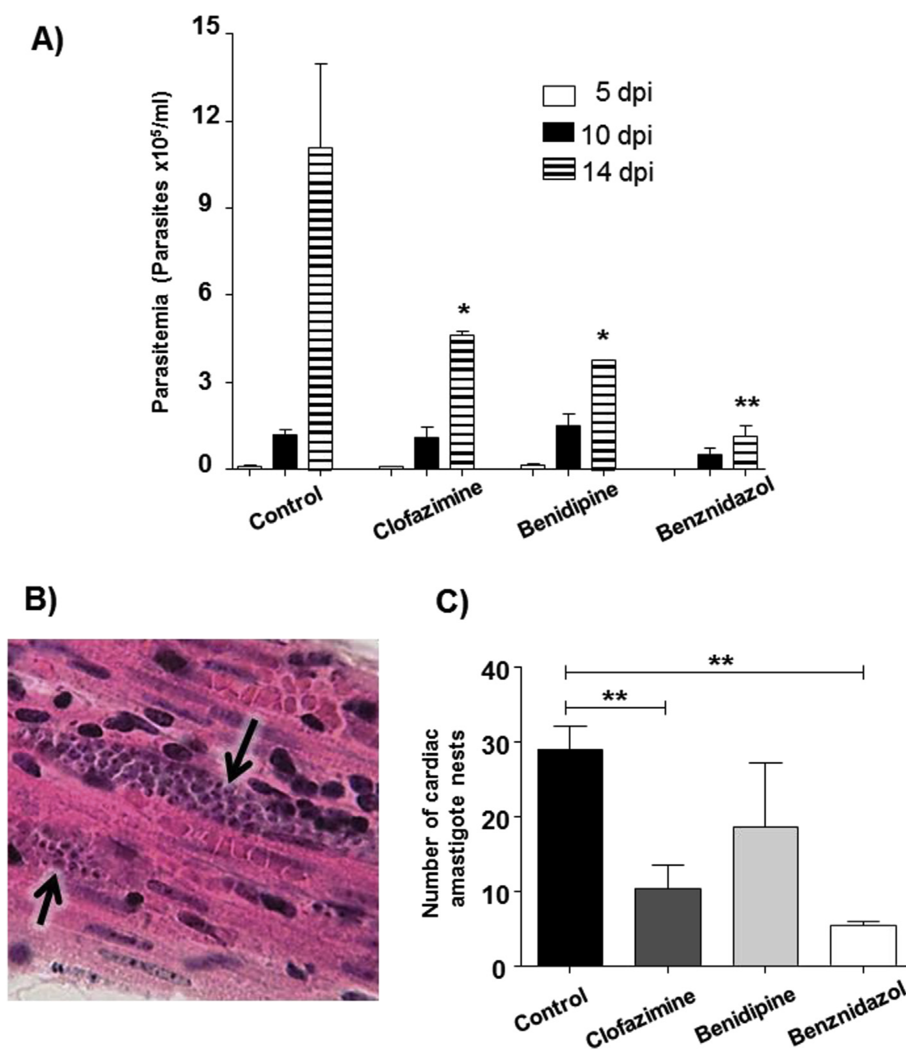

Fig. 8. Effects of clofazimine and benidipine on experimental *T. cruzi* acute infection in mice. Mice (3 animals per group) were infected with 1×10^5 TCT by IP injection and parasitemia was tested at 5 dpi. Animals with circulating parasites were treated daily with 1% DMSO in Saline solution alone (Control) or with the addition of Clofazimine, Benidipine or Benznidazole at the indicated dose according to described in *Materials and Methods*. Parasitemia was quantified at 5, 10, 14 and 20 dpi and results were expressed in parasite number/ml blood (A). Cardiac sections of infected animals were analyzed after HE staining to visualize amastigote nests (B). Number of *T. cruzi* nests quantified by conventional microscopy of cardiac tissues from each animal at the indicated treatment (C). Asterisks indicate significant differences from controls (*$p < 0.05$; **$p < 0.01$).

Analyzing the results from the cell monolayer experiments and the nature of the selected compounds, we have proposed a series of criteria to decide which of the candidates that showed a positive effect at the *in vitro* and *cellular* tests should progress to preclinical models. These criteria include: a) coincidence of the concentrations that show the effect at the *in vitro* and/or cellular level and the steady state concentration required (and tolerated) for the original therapeutic indication; b) possible additional beneficial effects on the progression or symptoms of the intended to treat disease emerging from the original indication (e.g. potential cardioprotective effects of benidipine on Chagas patients); c) side-effects profile (absence of a frequent side-effect that could exacerbate an intrinsic symptom of the intended to treat disease, e.g. cardiac side-effects in the case of Chagas).

After applying these criteria, we selected clofazimine and benidipine to move to a preclinical model of acute infection, where they also showed to be effective to lower parasitemia at doses much smaller than the one used for the positive control, benznidazole. It is worth to underline that our candidates were administered at doses five (clofazimine, 20 mg/kg/d) to ten (benidipine, 10 mg/kg/d) times lower than benznidazole (100 mg/kg/d). Thus, though none of both candidates have achieved a similar effect as benznidazole on the infection model, they showed a significant difference compared to the negative control, even by administrating very small doses compared to benznidazole. Remarkably, previous acute toxicity studies show that the LD_{50} (mice, po) for both candidates is much higher than the doses tested in the acute mice model of Chagas disease. Reported LD_{50}s for benidipine are above 300 mg/kg [83] while LD_{50} for clofazimine is 5 g/kg (USP Data sheet). Thus, administration of higher doses in the acute model of the infection should be considered in the near future assessing whether the candidates can achieve comparable or better results than benznidazole. It is also important to consider that benznidazole and nifurtimox are much more efficiently in the treatment of acute infection of *T. cruzi*, at least in the doses and prescriptions currently used. New experiments will be performed in the future to test the effect of Cz inhibitors in chronic models of infection. It should also be highlighted that one of the tested candidates, clofazimine, has not only proved to be effective in reducing the parasite levels in acute animal models of Chagas disease but also to reduce the

allocation of *T. cruzi* nests in cardiac tissue.

The present work successfully integrates computer-aided drug discovery with molecular and cellular biology and preclinical testing, confirming the utility of VS to develop knowledge-based drug repurposing. Such inter-disciplinary work optimizes, in terms of resources and time investment, the selection process of potential new therapeutics with a focus on drug repositioning. This is essential for the search of new medications for neglected diseases since such search is frequently developed through joint private-public initiatives, academy or public research institutions, with the consequent limitations on available funding and the need of translationally efficient approaches.

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Author contributions

As an interdisciplinary work, it has been developed as a three block work. The first block, the development of the computational model used in the VS campaign, was done by CLB, PHP, LG, JG, LBB and AT. The second block, the enzymatic and cellular assays, done by DB, CAL and CC. And the third block, the host cell infection assays and animal testing of the repurposed drugs, done by MCV, DB, AFC and PSR. All authors have actively and complementary contributed to the study design and results discussion.

Transparency declarations

None to declare.

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