Repurposing Strategy of Atorvastatin against *Trypanosoma cruzi*: *In Vitro* Monotherapy and Combined Therapy with Benznidazole Exhibit Synergistic Trypanocidal Activity


**ABSTRACT** Statins are inhibitors of cholesterol synthesis, but other biological properties, such as antimicrobial effects, have also been assigned to them, leading to their designation as pleiotropic agents. Our goal was to investigate the activity and selectivity of atorvastatin (AVA) against *Trypanosoma cruzi* by using *in vitro* models, aiming for more effective and safer therapeutic options through drug repurposing proposals for monotherapy and therapy in combination with benznidazole (BZ). Phenotypic screening was performed with different strains (Tulahuen [discrete typing unit (DTU) VI] and Y [DTU II]) and forms (intracellular forms, bloodstream trypomastigotes, and tissue-derived trypomastigotes) of the parasite. On assay of the Tulahuen strain, AVA was more active against intracellular amastigotes (selectivity index \(SI = 3\)). Also, against a parasite of another DTU (Y strain), this statin was more active (2.1-fold) and selective (2.4-fold) against bloodstream trypomastigotes (\(SI = 51\)) than against the intracellular forms (\(SI = 20\)). A cytomorphological approach using phalloidin-rhodamine permitted us to verify that AVA did not induce cell density reduction and that cardiac cells (CC) maintained their typical cytoarchitecture. Combinatory approaches using fixed-ratio methods showed that AVA and BZ gave synergistic interactions against both trypomastigotes and intracellular forms (mean sums of fractional inhibitory concentration indexes \(FICIs\) of 0.46 ± 0.12 and 0.48 ± 0.03, respectively). Thus, the repurposing strategy for AVA, especially in combination with BZ, which leads to a synergistic effect, is encouraging for future studies to identify novel therapeutic protocols for Chagas disease treatment.

**KEYWORDS** trypanocidal, Chagas disease, experimental chemotherapy, selectivity, statin, atorvastatin, HMGCR inhibitor, combined therapy

Statins are competitive hydroxy-3-methylglutaryl–coenzyme A (HMG-CoA) reductase inhibitors of cholesterol synthesis from the mevalonate pathway. These molecules also enhance the expression of low-density lipoprotein (LDL) receptors and increase the incorporation of LDL, leading to reduced serum levels of cholesterol (1). Several new roles have been assigned to statins, including some related to their hypcholesterolemic activity but also others not directly involved in this primary activity (2). These multitasking statin properties, known as pleiotropic effects, include antioxidative, anti-inflammatory, anti-atherogenic, and chemotherapeutic activities (3), suggesting the potential use of these drugs for prevention and treatment of infectious diseases, such as toxoplasmosis and malaria (4–8). Chagas disease (CD), caused by the protozoan intracellular parasite *Trypanosoma cruzi*, is an important public health problem that
affects more than 6 million people, with more than 14,000 annual deaths (9). Current therapeutic options for CD are limited to benznidazole (BZ) \((\text{N}-\text{benzyl-2-nitroimidazole acetamide})\) and nifurtimox (NF) \([3\text{-methyl}-4-(5\text{-nitrofurfurylideneamine})\text{ tetrahydro-4H-1, 4-tiazine-1, 1-dioxide}]\), and both display severe limitations, including side effects, lack of efficacy in the later, chronic phase, and the occurrence of naturally resistant strains (10).

Different therapeutic strategies have been used to identify new treatments that are more effective and safer, and several classes of compounds have been studied (9, 10). Among these are the inhibitors of the lipid biosynthetic route (11, 12), as sterols are essential components in biological membranes, exhibiting key roles in controlling fluidity, permeability, and ion channel functionality as well as in the cycles of cell division (13). In contrast to mammalian cells, which have cholesterol as a major sterol, protozoan agents such as \(T.\ cruzi\), like fungi, synthesize ergosterol endogenously, and therefore the inhibition of this biosynthetic pathway has been considered an important drug target for CD (11, 14). Unfortunately, clinical trials demonstrated a therapeutic failure of azole inhibitors of the CYP51 enzyme, although experimental models of \(T.\ cruzi\) infection \((\text{in vitro} \text{ and } \text{in vivo})\) have previously demonstrated promising preclinical findings (9).

Interestingly, Urbina et al. (15) reported that the antiparasitic effect of ketoconazole was improved by lovastatin (LOV) when murine experimental models of Chagas disease were used. Simvastatin (SIM) reduces heart inflammation and decreases the levels of serum and tissue inflammatory mediators in mouse models of acute \(T.\ cruzi\) infection (16). Also, SIM improved cardiac remodeling in \(T.\ cruzi\)-infected dogs (17), though it was not effective at reducing the peak parasitemia levels. Therefore, since Peña-Díaz et al. (18) found a functional HMG-CoA reductase in \(T.\ cruzi\), the goal of the present study was to evaluate the impact of drug repurposing schemes by using atorvastatin (AVA), the first synthetic statin, alone and in combination with the clinical drug for CD, i.e., BZ. The activity and mammalian toxicity of AVA and the cardiac cell (CC) viability in its presence were then assessed by use of different strains and forms of \(T.\ cruzi\), with consideration of selectivity among the distinct \(T.\ cruzi\) discrete typing units (DTUs), through well-standardized \textit{in vitro} approaches, in addition to exploration of its efficacy profile in combination with BZ.

### RESULTS

We started our analysis by evaluating the effect of AVA against trypomastigotes of \(T.\ cruzi\) \((\text{bloodstream trypomastigotes [BT] of strain Y, a representative of DTU II})\). Our findings demonstrated that AVA displayed a high trypanocidal effect \textit{in vitro}, exhibiting a 50% effective concentration \((\text{EC}_{50})\) of \(7.1 \pm 1.8 \mu M\) when BT forms were assayed and therefore being slightly more active than the reference drug \((\text{EC}_{50} = 13 \pm 2 \mu M)\) (Table 1). Regarding \textit{in vitro} cardiac toxicity, AVA demonstrated a low toxicity profile, presenting a 50% lethal concentration \((\text{LC}_{50})\) of \(360.7 \pm 18.2 \mu M\) after 24 h of compound exposure, and this low cardiotoxicity behavior led to high selectivity \((\text{SI} \text{ of } 51 \text{ for BT})\). Following our screening flowchart, we next evaluated the effect of AVA upon intracellular forms of the parasite, also exploring two different \(T.\ cruzi\) strains, belonging to DTUs VI and II. As shown in Table 2, after 48 h of incubation, AVA reached an \(\text{EC}_{50}\) of \(15 \pm 2.8 \mu M\) when CC were infected with the Y strain. Due to the high \(\text{LC}_{50}\)

### Table 1

<table>
<thead>
<tr>
<th>Drug</th>
<th>(\text{LC}_{50}) ((\mu M))</th>
<th>(\text{EC}_{50}) ((\mu M))</th>
<th>SI</th>
</tr>
</thead>
<tbody>
<tr>
<td>AVA</td>
<td>(360.7 \pm 18.2^b)</td>
<td>(7.1 \pm 1.8)</td>
<td>51</td>
</tr>
<tr>
<td>BZ</td>
<td>(&gt;1,000)</td>
<td>(13.0 \pm 2.0)</td>
<td>(&gt;77)</td>
</tr>
</tbody>
</table>

\(^a\)AVA, atorvastatin; BZ, benznidazole; \(\text{EC}_{50}\) 50% effective concentration; \(\text{LC}_{50}\) 50% lethal concentration; SI, selectivity index.

\(^b\)\(P < 0.01\) versus benznidazole by two-way ANOVA and Bonferroni’s post hoc test.
(320.0 ± 14.1 μM) of AVA after 48 h of exposure, the SI was also encouraging, reaching 21-fold (Table 2).

As shown in Fig. 1, CC incubated with AVA at the corresponding EC50 against intracellular forms did not exhibit toxic alterations in morphology and density (Fig. 1B) or cytoskeleton organization (Fig. 1D), instead displaying aspects similar to those of uninfected cells (Fig. 1E). AVA largely reduced the percentage of infected cells and the number of parasites per host cell compared to those for infected and untreated cultures (Fig. 1A and C). Next, as AVA was less potent against intracellular forms than against BT forms, we next assessed if AVA would have a similar behavior (less activity on intracellular forms) against other host cells infected with another parasite strain from another DTU. Infected L929 cell cultures infected with strain Tulahuen and exposed to nontoxic concentrations of AVA confirmed that this statin was less active against intracellular forms (EC50 = 25 μM), regardless of the parasite strain (Table 3). There

### Table 2

In vitro effects of AVA and BZ against intracellular amastigote forms of *T. cruzi* (Y strain) (EC50) and against cardiac muscle cell cultures (LC50) after treatment for 48 h, with corresponding selectivity indexes.

<table>
<thead>
<tr>
<th>Drug</th>
<th>LC50 (μM)</th>
<th>EC50 (μM) for indicated measure</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Infected cells</td>
</tr>
<tr>
<td>AVA</td>
<td>320.0 ± 14.1</td>
<td>12.3 ± 2.1</td>
</tr>
<tr>
<td>BZ</td>
<td>&gt;1,000</td>
<td>ND</td>
</tr>
</tbody>
</table>

AVA, atorvastatin; BZ, benznidazole; EC50, 50% effective concentration; LC50, 50% lethal concentration; SI, selectivity Index; ND, not determined.

Data from reference 39.
were no statistical differences (P > 0.05) between EC\textsubscript{50} values against intracellular forms of strains Tulahuen and Y.

The next step was to investigate if the association of AVA with BZ could improve the trypanocidal effect upon intracellular and trypomastigote forms of \textit{T. cruzi}. The \textit{in vitro} interaction of AVA and BZ was evaluated, and mean fractional inhibitory concentration indexes (FICIs) and representative isobolograms are presented in Fig. 2. The findings show an important leftward shift of the combined-therapy curves for both BZ (Fig. 2a) and AVA (Fig. 2b) compared to the curves for monotherapy. A similar behavior was also noticed when BT were assayed (Fig. 2d), with a leftward shift for AVA combination therapy (Fig. 2e) compared to the monotherapy curve. The interaction of AVA and BZ was classified as synergistic against both BT (Fig. 2c) and intracellular forms (Fig. 2f), presenting mean \( \Sigma \) FICIs equal to 0.46 ± 0.12 and 0.48 ± 0.03, respectively.

**DISCUSSION**

According to Don and Ioset (19), the characteristics of a hit compound for anti-\textit{T. cruzi} drug screening include an EC\textsubscript{50} value of up to 10 \( \mu \)M against intracellular forms

### Table 3

<table>
<thead>
<tr>
<th>Drug</th>
<th>LC\textsubscript{50} (( \mu )M)</th>
<th>% of parasite kill at 10 ( \mu )M</th>
<th>EC\textsubscript{50} (( \mu )M)</th>
<th>SI</th>
</tr>
</thead>
<tbody>
<tr>
<td>AVA</td>
<td>76.1 ± 2.2</td>
<td>31.7 ± 5.8</td>
<td>25.3 ± 3.1</td>
<td>3</td>
</tr>
<tr>
<td>BZ</td>
<td>169.1 ± 27.2</td>
<td>87.8 ± 7.1</td>
<td>1.8 ± 0.7</td>
<td>92</td>
</tr>
</tbody>
</table>

AVA, atorvastatin; BZ, benznidazole; EC\textsubscript{50}, 50\% effective concentration; LC\textsubscript{50}, 50\% lethal concentration; SI, selectivity index.

![Image](http://aac.asm.org/)

**FIG 2** In vitro trypanocidal combinatory analysis of atorvastatin (AVA) and benznidazole (BZ) against bloodstream trypomastigotes (Y strain) (a to c) and intracellular forms (Tulahuen strain) (d to f). Dose-response curves are shown for benznidazole (BZ) (a and d), atorvastatin (AVA) (b and e), and both (at the indicated fixed-ratio combinations) (c and f). Representative isobolograms of \textit{in vitro} interaction of AVA and BZ against \textit{T. cruzi}. The EC\textsubscript{50} of AVA is plotted on the abscissa, and the EC\textsubscript{50} of BZ is plotted on the ordinate. The plotted points are the EC\textsubscript{50}s for fixed ratios (5:0, 4:1, 3:2, 2:3, 1:4, and 0:5) of AVA and BZ. \( \Sigma \) FICIs of <0.5 indicate synergism, \( \Sigma \) FICIs of >0.5 and ≤4.0 indicate additivity, and \( \Sigma \) FICIs of >4.0 indicate antagonism.
and an SI of at least 10, in addition to the absence of structural alerts of safety concerns (such as genotoxicity) and the presence of druggability properties, at least as evaluated using in silico platforms. Also, the target product profile (TPP) for CD recommends analysis against both relevant parasite forms for mammalian hosts (trypomastigotes and intracellular forms) and examination of the effects on representatives of different T. cruzi DTUs (20).

Although de Souza and Rodrigues (21) suggested AVA as a promising ergosterol biosynthesis inhibitor (EBI) in their 2009 review of sterol pathways as targets for antitrypanosomatid drugs, few studies have been performed to test the effects of statins on animal T. cruzi infection, and they revealed controversial data (17, 22). Thus, due to the well-known pleiotropic profile of AVA acting as an antiparasitic agent, we conducted a repurposing study using this molecule, taking into consideration the phenotypic steps suggested for a novel anti-T. cruzi entity (23). The repurposing approach is largely encouraged for drug discovery for neglected pathologies, such as CD, since it may reduce costs and time due to the use of drugs that are already approved for one purpose for treatment of other clinical conditions (10, 23). Concerning the safety aspects, as demonstrated through preclinical and clinical toxicological screenings performed by Ciavavino et al. (24), AVA is not mutagenic or genotoxic and is considered a safe drug currently on the market. Regarding its potential application as an antiparasitic drug, Carvalho et al. (25) showed that the AVA pyrrole region hybridized with quinolines was effective against Plasmodium falciparum in parasitized red blood cells, suggesting that the pyrrole ring of this molecule has a central role in its microbicidal effect, possibly acting as a secondary pharmacophoric region. As suggested previously by Kaiser et al. (26), due to the large data bank of antimalarial agents, the repurposing of hit-to-lead compounds merits further screening against other kinetoplastid diseases, including CD. Thus, our present study evaluated the potential activity of AVA as an anti-T. cruzi agent by using a well-standardized pipeline for CD (23).

Our findings demonstrated a promising effect of AVA when T. cruzi was assessed, especially toward trypomastigotes, against which AVA gave considerable selectivity indexes (>50), which is a desirable characteristic for a novel hit drug for CD (20). Another aspect is that AVA displayed a low cardiotoxicity profile, which is a favorable characteristic since the heart is one of the main targets of T. cruzi infection and inflammation in CD (12). Our data corroborate in part the literature data demonstrating the beneficial cardiovascular pleiotropic effect of statins, such as AVA (2, 3, 22).

Florin-Christensen et al. (27) and Melo et al. (17) demonstrated the activities of lovastatin and SIM, respectively, against epimastigotes of T. cruzi (strain Y). However, this form is not the relevant one for assaying the effects of novel drugs for CD, since epimastigotes are the proliferative forms found only in the vector and thus are not adequate for exploring the activity and selectivity of novel anti-T. cruzi agents (23).

It is important that trypomastigotes were consistently more vulnerable (2- to 3-fold) than intracellular forms to treatment with AVA. This differential susceptibility according to the parasite form of T. cruzi may be due to the intracellular cytoplasmic microenvironment localization of amastigotes. It is possible that the plasma membranes of parasites are more permeable to AVA than the membranes of their mammalian hosts, reducing the access of free AVA directly to the intracellular parasites (e.g., because of the host plasma membrane barrier). Thus, as an alternative to increase the potency of AVA against intracellular forms, the use of a drug carrier and a novel formulation may allow for a higher rate of permeation across the host cell and/or reduce drug efflux.

Another hypothesis regards differences related to the parasite stage which merit further exploration, including (i) localization in distinct microenvironments (blood versus intracellular milieu), with differential lipid levels and access, and (ii) distinct metabolism, uptake, or efflux levels among the nonproliferative versus highly multiplicative parasite forms, among others.

A remarkable present result was achieved using combinatory schemes resulting in FICI values of <0.5, indicating synergism with the reference drug for CD, benznida-
zole. Our data corroborate previous studies that used combinatorial repurposing approaches (AVA and a reference drug, such as quinine) on several strains of *Plasmodium falciparum* and reported a synergistic profile (6).

In practice, *de novo* drug discovery and development are very long and costly processes, so the repurposing strategy has been stimulated largely as an alternative for treatment of several orphan pathologies, since the steps related to drug pharmacokinetics and safety profiles are approved by the regulatory authorities (25). Drug repurposing works side by side with the possibility of combining drugs, which is already the established treatment protocol for some infectious diseases, such as tuberculosis and malaria (28). Recently, the repurposing of NF and its combination with efloornithine to treat African trypanosomiasis were made available and demonstrated positive outcomes (29, 30). The combination of licensed drugs enables cost reduction and reduces side effects, as well as providing a wider arsenal against neglected diseases, improved efficacy, and, finally, possible changes in posology and administration which encourage patient adherence to treatment (31). In addition, this strategy allows the attack of different targets in the parasite as well as the potential bypass or even avoidance of drug resistance and has been claimed to be an alternative for CD therapy (31–33).

The development of novel alternatives for Chagas disease therapy still represents an urgent issue (34). The therapeutic failure of EBIs, such as posaconazole and ravucona-zole, in clinical trials for CD, along with the withdrawal of the nitro derivative fenini-dazole from recent clinical trials due to its low safety aspects for CD patients, justifies the search for alternative anti-*T. cruzi* molecules as well as novel therapeutic approaches (20). Note that a hybrid molecule derived from the EBI ravuconazole (E1224) recently provided satisfactory results in a clinical trial with chronic CD patients in Colombia (35).

The bulk of our data suggest that the use of statins in combination with BZ to treat parasitic infections is a promising therapeutic approach, and they also justify the synthesis of novel statin molecules more selective for parasite enzymes, with the aim of contributing to the discovery of novel alternative therapeutic options for parasitic pathologies. Our combined-therapy results are thus very encouraging, showing a synergistic effect between AVA and BZ that improves the efficacy and potency of both drugs in combination against both trypomastigotes and intracellular forms of *T. cruzi* strains belonging to different parasite DTUs which are relevant for human infection.

**MATERIALS AND METHODS**

**Compounds.** For analysis of the effects of compounds against *T. cruzi* and upon mammalian viability, stock solutions of AVA (FarManguinhos, Fiocruz, Brazil) were prepared in dimethyl sulfoxide (DMSO), with the final concentration of the solvent never exceeding 0.6%, which does not exert any toxicity (data not shown). BZ (Laboratório Farmacêutico do Estado de Pernambuco [LAFEPE], Brazil) was used as a trypanocidal reference drug control, and aliquots were stored at −20°C.

**Cell cultures.** Mouse L929 fibroblasts obtained from the American Type Culture Collection (Manas-sas, VA) were cultured at 37°C in RPMI 1640 medium (Gibco BRL) supplemented with 10% fetal bovine serum (FBS) and 2 mM glutamine, as reported by Timm et al. (36). For both drug cytotoxicity and infection assays, primary cultures of cardiac cells (CC) were obtained as reported by Batista et al. (37). The cultures were sustained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 6% FBS, 2.5 mM CaCl₂, 1 mM l-glutamine, and 2% chicken embryo extract and were maintained at 37°C in an atmosphere of 5% CO₂ and air.

**Parasites.** Tissue culture-derived trypomastigotes (CT) from strain Tulahuen (expressing the *Esch-erichia coli* β-galactosidase gene) were maintained in L929 cell lines. After 96 h of parasite infection, the supernatant, enriched with released trypomastigotes, was collected and the parasite purified following a previously established protocol (36). Bloodstream trypomastigotes (BT) of the Y strain were obtained from blood samples of infected albino Swiss mice at the peak of parasitemia, and the purified parasites were resuspended in RPMI medium as reported previously (38). Intracellular amastigotes of strains Y and Tulahuen were also evaluated. For the analysis using the Y strain, CC were infected with BT for 24 h (parasite/CC ratio of 10:1), rinsed to remove noninternalized parasites, and further incubated for another 48 h with increasing but nontoxic doses of the test compound (37). For the Tulahuen strain, L929 cell cultures were infected for 2 h with CT (10:1 ratio), and then the cultures were rinsed to get rid of the noninternalized parasites. Next, the cell cultures were further incubated for another 24 h at 37°C, and then nontoxic compound concentrations were added and the cultures monitored for 96 h (36).

**In vitro tests of mammalian cytotoxicity.** CC were incubated for 24 to 48 h at 37°C with different concentrations of AVA (up to 500 μM) diluted in DMEM (without phenol red), and then their morphology...
Fluorescence cytoarchitecture arrangement assay. In an attempt to assess cardiac cell viability through use of a contractility apparatus, the subcellular cytoskeleton configuration was checked. The cultures were treated for 48 h with AVA at the EC_{50} obtained against intracellular forms (15 μM) (Table 2) and then washed and stained with 800 μg/ml phalloidin-rhodamine and 1 μg/ml 4’,6-diamidino-2-phenylindole (DAPI) for actin filament and DNA visualization, respectively (42). Doxorubicin (10 μM) was used as a cytoarchitecture derangement induction control, and 2.5 μM BZ was used as a T. cruzi infection positive control (43). The samples were observed by use of a Zeiss microscope equipped with epifluorescence visualization capability.

Ethics. All procedures were carried out in accordance with the guidelines established by the FiOCRUZ Committee of Ethics for the Use of Animals (CEUA LW16/14).

ACKNOWLEDGMENTS

We thank the National Council of P&D of Brazil (CNPq) for the fellowship granted for this work. We also thank the Foundation for Research of Rio de Janeiro State (FAPERJ),
REFERENCES


