AMERICAN SOCIETY FOR MICROBIOLOGY | Antimicrobial Agents and Chemotherapy®

EXPERIMENTAL THERAPEUTICS

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

C. F. Araujo-Lima,[a,b] R. B. Peres,[a] P. B. Silva,[a] M. M. Batista,[a] C. A. F. Aiub,[c] I. Felzenszwalb,[b] M. N. C. Soeiro[a]

[a]Laboratório de Biologia Celular, Instituto Oswaldo Cruz, Fundação Oswaldo Cruz (FIOCRUZ), Rio de Janeiro, Brazil

[b]Departamento de Biofísica e Biometria, Instituto de Biologia Roberto Alcantara Gomes, Universidade do Estado do Rio de Janeiro (UERJ), Rio de Janeiro, Brazil

[c]Departamento de Genética e Biologia Molecular, Instituto Biomédico, Universidade Federal do Estado do Rio de Janeiro (UNIRIO), Rio de Janeiro, Brazil

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 induced 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 hypocholesterolemic activity but also others not directly involved in this primary activity (2). These multitasking statin properties, known as pleiotropic effects, include antioxidative, anti-inflammatory, antiatherogenic, 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

Received 14 May 2018 **Accepted** 22 June 2018

Accepted manuscript posted online 9 July 2018

Citation Araujo-Lima CF, Peres RB, Silva PB, Batista MM, Aiub CAF, Felzenszwalb I, Soeiro MNC. 2018. Repurposing strategy of atorvastatin against *Trypanosoma cruzi*: *in vitro* monotherapy and combined therapy with benznidazole exhibit synergistic trypanocidal activity. Antimicrob Agents Chemother 62:e00979-18. https://doi.org/10.1128/AAC.00979-18.

Address correspondence to M. N. C. Soeiro, soeiro@ioc.fiocruz.br.

TABLE 1 *In vitro* effects of AVA and BZ against trypomastigote forms of *T. cruzi* (Y strain BT) (EC_{50}) and against cardiac muscle cell cultures (LC_{50}) after 24 h of treatment, with corresponding selectivity indexes[a]

Drug	LC_{50} (μM)	EC_{50} (μM)	SI
AVA	360.7 ± 18.2[b]	7.1 ± 1.8	51
BZ	>1,000	13.0 ± 2.0	>77

[a]AVA, atorvastatin; BZ, benznidazole; EC_{50}, 50% effective concentration; LC_{50}, 50% lethal concentration; SI, selectivity index.
[b]$P < 0.01$ versus benznidazole by two-way ANOVA and Bonferroni's *post hoc* test.

affects more than 6 million people, with more than 14,000 annual deaths (9). Current therapeutic options for CD are limited to benznidazole (BZ) (*N*-benzyl-2-nitroimidazole acetamide) and nifurtimox (NF) [3-methyl-4-(5′-nitrofurfurylideneamine) 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 (*in vitro* and *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 *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* (bloodstream trypomastigotes [BT] of strain Y, a representative of DTU II). Our findings demonstrated that AVA displayed a high trypanocidal effect *in vitro*, exhibiting a 50% effective concentration (EC_{50}) of 7.1 ± 1.8 μM when BT forms were assayed and therefore being slightly more active than the reference drug (EC_{50} = 13 ± 2 μM) (Table 1). Regarding *in vitro* cardiac toxicity, AVA demonstrated a low toxicity profile, presenting a 50% lethal concentration (LC_{50}) of 360.7 ± 18.2 μM after 24 h of compound exposure, and this low cardiotoxicity behavior led to high selectivity (selectivity index [SI] of 51 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 EC_{50} of 15 ± 2.8 μM when CC were infected with the Y strain. Due to the high LC_{50}

TABLE 2 *In vitro* effects of AVA and BZ against intracellular amastigote forms of *T. cruzi* (Y strain) (EC_{50}) and against cardiac muscle cell cultures (LC_{50}) after treatment for 48 h, with corresponding selectivity indexes[a]

Drug	LC_{50} (μM)	EC_{50} (μM) for indicated measure			SI
		Infected cells	Parasites per cell	Infection index	
AVA	320.0 ± 14.1	12.3 ± 2.1	7.3 ± 1.7	15.0 ± 2.8	21
BZ[b]	>1,000	ND	ND	2.8 ± 1.9	>357

[a]AVA, atorvastatin; BZ, benznidazole; EC_{50}, 50% effective concentration; LC_{50}, 50% lethal concentration; SI, selectivity Index; ND, not determined.
[b]Data from reference 39.

(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 EC_{50} 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 (EC_{50} = 25 μM), regardless of the parasite strain (Table 3). There

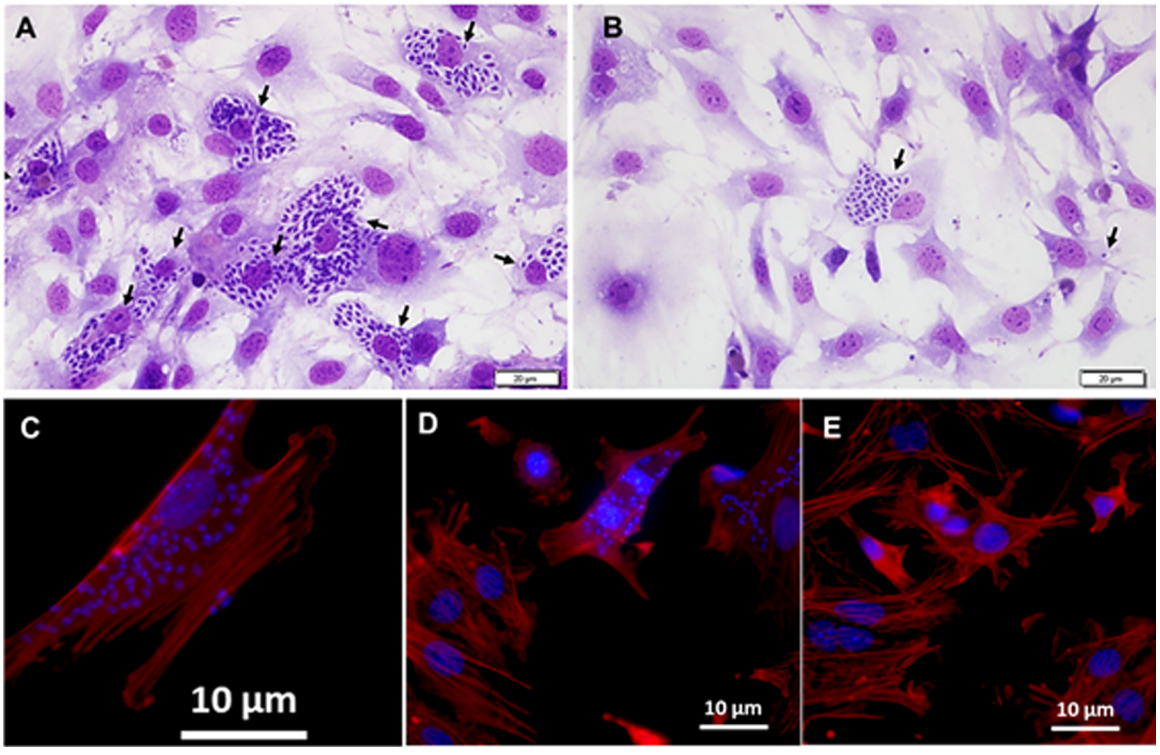

FIG 1 Activity of AVA against intracellular forms of *T. cruzi* (strain Y) after treatment for 48 h at 37°C. (A) *T. cruzi*-infected cardiac cells incubated with vehicle. (B) *T. cruzi*-infected cardiac cells incubated with AVA at the EC_{50} (15.0 μM). The arrows point to intracellular parasites. The cells were stained with Giemsa stain. (C) *T. cruzi*-infected cardiac cells. (D) *T. cruzi*-infected cardiac cells incubated with AVA at the EC_{50} (15.0 μM). (E) Uninfected, untreated cardiac cells. The epifluorescence micrographs show cells labeled with phalloidin-rhodamine (red) and DAPI (blue).

TABLE 3 *In vitro* effects of AVA and BZ against intracellular forms of *T. cruzi* (strain Tulahuen transfected with β-galactosidase) (EC_{50}) and L929 cell cultures (LC_{50}) after 96 h of treatment, with corresponding selectivity indexes[a]

Drug	LC_{50} (μM)	% of parasite kill at 10 μM	EC_{50} (μM)	SI
AVA	76.1 ± 2.2	31.7 ± 5.8	25.3 ± 3.1	3
BZ	169.1 ± 27.2	87.8 ± 7.1	1.8 ± 0.7	92

[a]AVA, atorvastatin; BZ, benznidazole; EC_{50}, 50% effective concentration; LC_{50}, 50% lethal concentration; SI, selectivity index.

were no statistical differences ($P > 0.05$) between EC_{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 *T. cruzi*. The *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 ∑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-*T. cruzi* drug screening include an EC_{50} value of up to 10 μM against intracellular forms

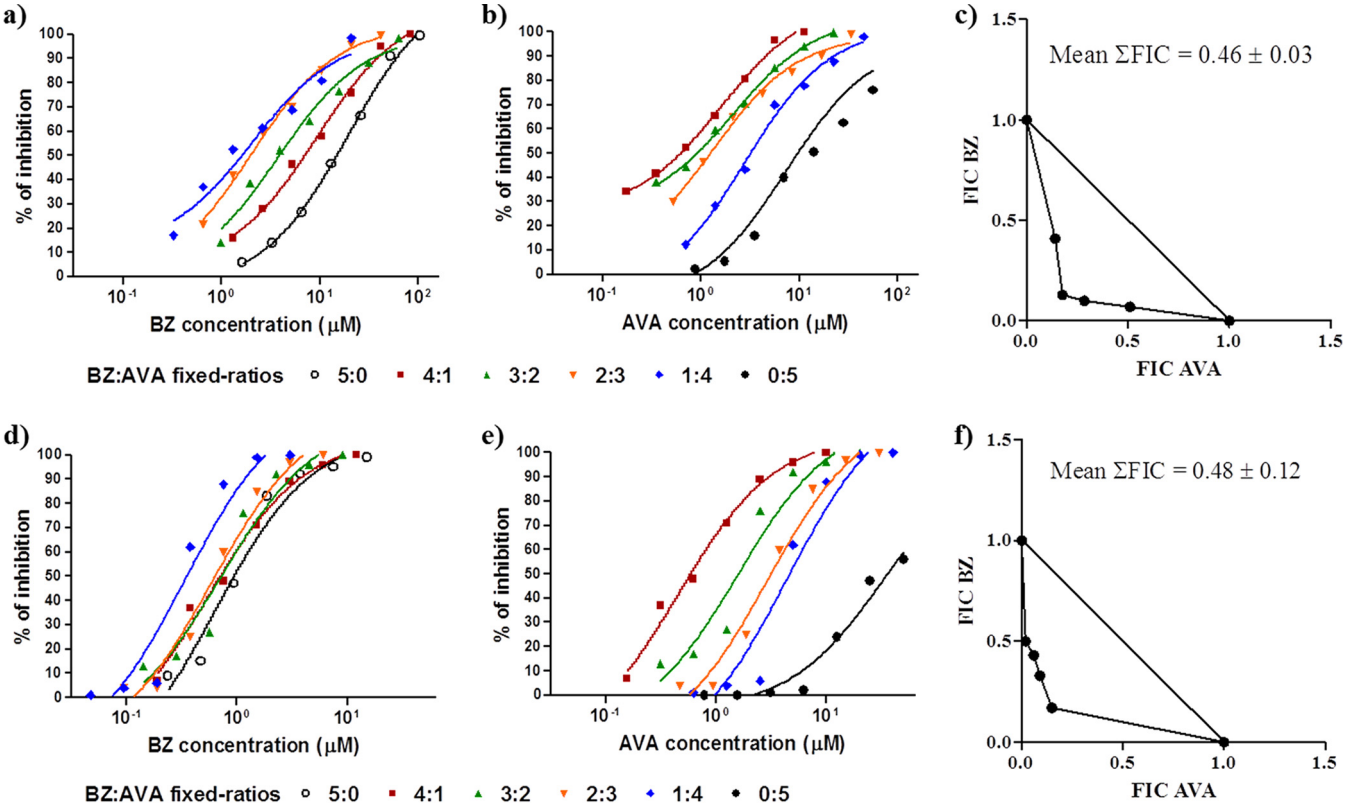

FIG 2 *In vitro* trypanocidal combinatory analysis of atorvastatin (AVA) and benznidazole (BZ) against bloodstream trypomastigotes (Y strain) (a to c) and intracellular forms (Tulahuen strain) (d to f). Dose-response curves are shown for benznidazole (BZ) (a and d), atorvastatin (AVA) (b and e), and both (at the indicated fixed-ratio combinations). (c and f) Representative isobolograms of *in vitro* interaction of AVA and BZ against *T. cruzi*. The EC_{50} of AVA is plotted on the abscissa, and the EC_{50} of BZ is plotted on the ordinate. The plotted points are the EC_{50}s for fixed ratios (5:0, 4:1, 3:2, 1:4, and 0:5) of AVA and BZ. ∑FICIs of <0.5 indicate synergism, ∑FICIs of >0.5 and ≤4.0 indicate additivity, and ∑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 Ciaravino 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 $\sum$FICI values of $<$0.5, indicating synergism with the reference drug for CD, benznida-

zole. Our data corroborate previous studies that used combinatory 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 eflornithine 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 ravuconazole, in clinical trials for CD, along with the withdrawal of the nitro derivative fexinidazole 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 (Manassas, 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 $CaCl_2$, 1 mM L-glutamine, and 2% chicken embryo extract and were maintained at 37°C in an atmosphere of 5% CO_2 and air.

Parasites. Tissue culture-derived trypomastigotes (CT) from strain Tulahuen (expressing the *Escherichia 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

and spontaneous contractibility were evaluated by light microscopy and their cellular viability determined by the PrestoBlue assay. For this colorimetric bioassay, 10 μl PrestoBlue (Invitrogen) was added to each well, and the plate was further incubated for 5 h. A similar protocol was used to evaluate L929 cell viability after 96 h of exposure to the compounds, using 10 μl alamarBlue (Invitrogen). For negative controls, the alamarBlue assay was also performed without mammalian cells, running only DMEM and DMEM containing AVA and BZ (at higher concentrations). The absorbance was determined (at 570 and 600 nm), and the results were expressed as percentages of reduction between compound- and vehicle-treated cell cultures, following the manufacturer's instructions. The LC_{50}, which corresponds to the concentration that reduces cellular viability by 50%, was then calculated as reported previously (36).

Trypanocidal analysis. BT forms of the Y strain (5×10^6 per ml) were incubated for up to 24 h at 37°C in RPMI medium in the presence or absence of serial dilutions of AVA (0 to 32 μM) and BZ (up to 100 μM). After incubation with the compound, the parasite death rates were determined by light microscopy via direct quantification of the number of live parasites by use of a Neubauer chamber, and the EC_{50} (compound concentration that reduces the number of parasites by 50%) was calculated (37). For the assay of intracellular forms, strains Y and Tulahuen were employed along with CC and L929 cells, respectively, as mentioned above. Briefly, Tulahuen-infected L929 cultures were exposed to serially diluted nontoxic concentrations of AVA diluted in RPMI medium to determine the EC_{50} (39). After 96 h of compound incubation at 37°C, chlorophenol red glycoside (500 μM) in 0.5% Nonidet P-40 was added to each well, and the plate was incubated for 18 h at 37°C. Next, the absorbance was measured at 570 nm. Uninfected and *T. cruzi*-infected cultures subjected to only vehicle and BZ exposure were run in parallel. The results are expressed as percentages of parasite growth inhibition in compound-tested cells compared to that in infected cells and untreated cells (38). Triplicate samples were run in the same plate, and at least two assays were performed for each analysis. For analysis of the effect against intracellular amastigotes of the Y strain, after 24 h of parasite-host cell interaction, the infected CC were washed to remove free parasites and then incubated for another 48 h with increasing concentrations of the test compounds. CC were maintained at 37°C in an atmosphere of 5% CO_2 and air, and the medium was replaced every 24 h. Untreated and treated infected CC were fixed and stained with Giemsa solution, and the mean numbers of infected host cells and of parasites per infected cell were scored as reported previously (39). Only characteristic *T. cruzi* nuclei and kinetoplasts were counted as surviving parasites, since irregular structures may indicate parasites undergoing death. The compound activity was estimated by calculating the infection index (II) (percentage of infected cells times average number of intracellular amastigotes per infected host cell) (37). At least two assays were performed in duplicate for each analysis. Statistical analysis was performed by analysis of variance (ANOVA) as reported by De Araújo et al. (39). All *in vitro* assays were run at least twice in triplicate, and statistical analysis was performed by two-way ANOVA and Bonferroni's *post hoc* test, with P values of <0.01 considered significant.

Determination of drug interactions against BT and intracellular forms of *T. cruzi*. *In vitro* drug interactions were assessed using a fixed-ratio method (28) for combining AVA and BZ. In these assays, predetermined EC_{50}s were used to determine the top concentrations of the individual drugs to ensure that the EC_{50} fell near the midpoint of a six-point 2-fold dilution series. The top concentrations used were 100 μM for BZ and 50 μM for AVA in a 24-h assay (against strain Y BT) and 15 μM for BZ and 50 μM for AVA in a 96-h assay (against strain Tulahuen intracellular forms). The top concentrations were used to prepare AVA and BZ solutions at fixed ratios of 5:0, 4:1, 3:2, 2:3, 1:4, and 0:5, as reported previously (40, 41).

Determination of FICIs, isobologram construction, and classification of the nature of the interaction. Fractional inhibitory concentration indexes (FICIs) were calculated as follows: FICI of AVA = EC_{50} of AVA in combination/EC_{50} of AVA alone. The same equation was applied to the partner drug (BZ). The sum of the FICIs ($\sum$FICI) was calculated as the FICI of AVA plus the FICI of BZ. An overall mean $\sum$FICI was calculated for each combination and used to classify the nature of the interaction. Isobolograms were constructed by plotting the AVA EC_{50} against the BZ EC_{50}. Statistical analysis was performed individually for each assay by using an analysis of variance program, and the level of significance was set at a P value of ≤ 0.05. Interactions were categorized as described by Simões-Silva et al. (38), with synergism classified by a mean $\sum$FICI of ≤ 0.5, antagonism classified by a mean $\sum$FICI of >4.0, and indifference (or an additive effect) classified by a mean $\sum$FICI of >0.5 and ≤ 4.0. All assays were conducted in triplicate, and the results are averages for at least three independent experiments (38).

Fluorescence cytoarchitecture arrangement assay. In an attempt to assess cardiac cell viability through use of a contractibility 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),

FIOCRUZ PAEF, and CNPq for financial support. M.N.C.S. and I.F. are research fellows of CNPq. C.F.A.-L. is a FAPERJ Nota 10 Ph.D. student. M.N.C.S. and I.F. are CNE researchers.

We thank Nubia Boechat for kindly providing atorvastatin.

REFERENCES

1. Grip O, Janciauskiene S, Lindgren S. 2002. Atorvastatin activates PPAR-gamma and attenuates the inflammatory response in human monocytes. Inflamm Res 51:58–62. https://doi.org/10.1007/BF02684000.
2. Liao JK, Laufs U. 2005. Pleiotropic effects of statins. Annu Rev Pharmacol Toxicol 45:89–118. https://doi.org/10.1146/annurev.pharmtox.45.120403.095748.
3. Davignon J. 2004. Beneficial cardiovascular pleiotropic effects of statins. Circulation 109:III-39–III-43.
4. Cortez E, Stumbo AC, Oliveira M, Barbosa HS, Carvalho L. 2009. Statins inhibit *Toxoplasma gondii* multiplication in macrophages in vitro. Int J Antimicrob Agents 33:185–186. https://doi.org/10.1016/j.ijantimicag.2008.07.026.
5. Parquet V, Briolant S, Torrentino-Madamet M, Henry M, Almeras L, Amalvict R, Baret E, Fusaï T, Rogier C, Pradines B. 2009. Atorvastatin is a promising partner for antimalarial drugs in treatment of Plasmodium falciparum malaria. Antimicrob Agents Chemother 53:2248–2252. https://doi.org/10.1128/AAC.01462-08.
6. Parquet V, Henry M, Wurtz N, Dormoi J, Briolant S, Gil M, Baret E, Amalvict R, Rogier C, Pradines B. 2010. Atorvastatin as a potential anti-malarial drug: in vitro synergy in combinational therapy with quinine against *Plasmodium falciparum*. Malar J 9:139. https://doi.org/10.1186/1475-2875-9-139.
7. Dormoi J, Briolant S, Desgrouas C, Pradines B. 2013. Impact of methylene blue and atorvastatin combination therapy on the apparition of cerebral malaria in a murine model. Malar J 12:127. https://doi.org/10.1186/1475-2875-12-127.
8. Dormoi J, Savini H, Amalvict R, Baret E, Pradines B. 2014. In vitro interaction of lumefantrine and piperaquine by atorvastatin against Plasmodium falciparum. Malar J 13:189. https://doi.org/10.1186/1475-2875-13-189.
9. Pérez-Molina JA, Perez AM, Norman FF, Monge-Maillo B, López-Vélez R. 2015. Old and new challenges in Chagas disease. Lancet Infect Dis 15:1347–1356. https://doi.org/10.1016/S1473-3099(15)00243-1.
10. Soeiro MN, de Castro SL. 2009. *Trypanosoma cruzi* targets for new chemotherapeutic approaches. Expert Opin Ther Targets 13:105–121. https://doi.org/10.1517/14728220802623881.
11. Urbina JA. 2009. Ergosterol biosynthesis and drug development for Chagas disease. Mem Inst Oswaldo Cruz 104(Suppl 1):S311–S318. https://doi.org/10.1590/S0074-02762009000900041.
12. Soeiro MDN, de Souza EM, da Silva CF, Batista DDG, Batista MM, Pavão BP, Araújo JS, Aiub CA, da Silva PB, Lionel J, Britto C, Kim K, Sulikowski G, Hargrove TY, Waterman MR, Lepesheva GI. 2013. In vitro and in vivo studies of the antiparasitic activity of sterol 14α-demethylase (CYP51) inhibitor VNI against drug-resistant strains of Trypanosoma cruzi. Antimicrob Agents Chemother 57:4151–4163. https://doi.org/10.1128/AAC.00070-13.
13. Lepesheva GI, Ott RD, Hargrove TY, Kleshchenko YY, Schuster I, Nes WD, Hill GC, Villalta F, Waterman MR. 2007. Sterol 14alpha-demethylase as a potential target for antitrypanosomal therapy: enzyme inhibition and parasite cell growth. Chem Biol 14:1283–1293. https://doi.org/10.1016/j.chembiol.2007.10.011.
14. Lepesheva GI, Villalta F, Waterman MR. 2011. Targeting Trypanosoma cruzi sterol 14α-demethylase (CYP51). Adv Parasitol 75:65–87. https://doi.org/10.1016/B978-0-12-385863-4.00004-6.
15. Urbina JA, Lazardi K, Marchan E, Visbal G, Aguirre T, Piras MM, Piras R, Maldonado RA, Payares G, de Souza W. 1993. Mevinolin (lovastatin) potentiates the antiproliferative effects of ketoconazole and terbinafine against *Trypanosoma* (*Schizotrypanum*) *cruzi*: *in vitro* and *in vivo* studies. Antimicrob Agents Chemother 37:580–591. https://doi.org/10.1128/AAC.37.3.580.
16. Silva RR, Shrestha-Bajracharya D, Almeida-Leite CM, Leite R, Bahia MT, Talvani A. 2012. Short-term therapy with simvastatin reduces inflammatory mediators and heart inflammation during the acute phase of experimental Chagas disease. Mem Inst Oswaldo Cruz 107:513–521. https://doi.org/10.1590/S0074-02762012000400012.
17. Melo L, Caldas IS, Azevedo MA, Gonçalves KR, da Silva do Nascimento AF, Figueiredo VP, de Figueiredo Diniz L, de Lima WG, Torres RM, Bahia MT, Talvani A. 2011. Low doses of simvastatin therapy ameliorate cardiac inflammatory remodeling in Trypanosoma cruzi-infected dogs. Am J Trop Med Hyg 84:325–331. https://doi.org/10.4269/ajtmh.2011.10-0451.
18. Peña-Díaz J, Montalvetti A, Camacho A, Gallego C, Ruiz-Perez LM, Gonzalez-Pacanowska D. 1997. A soluble 3-hydroxy-3-methylglutaryl-CoA reductase in the protozoan *Trypanosoma cruzi*. Biochem J 324:619–626. https://doi.org/10.1042/bj3240619.
19. Don R, Ioset JR. 2014. Screening strategies to identify new chemical diversity for drug development to treat kinetoplastid infections. Parasitology 141:140–146. https://doi.org/10.1017/S003118201300142X.
20. Chatelain E, Konar N. 2015. Translational challenges of animal models in Chagas disease drug development: a review. Drug Des Devel Ther 9:4807–4823. https://doi.org/10.2147/DDDT.S90208.
21. de Souza W, Rodrigues JC. 2009. Sterol biosynthesis pathway as target for anti-trypanosomatid drugs. Interdiscip Perspect Infect Dis 2009:642502. https://doi.org/10.1155/2009/642502.
22. Zhao D, Lizardo K, Cui MH, Ambadipudi K, Lora J, Jelicks LA, Nagajyothi JF. 2016. Antagonistic effect of atorvastatin on high fat diet induced survival during acute Chagas disease. Microbes Infect 18:675–686. https://doi.org/10.1016/j.micinf.2016.06.006.
23. Romanha AJ, Castro SL, Soeiro MDN, Lannes-Vieira J, Ribeiro I, Talvani A, Bourdin B, Blum B, Olivieri B, Zani C, Spadafora C, Chiari E, Chatelain E, Chaves G, Calzada JE, Bustamante JM, Freitas-Junior LH, Romero LI, Bahia MT, Lotrowska M, Soares M, Andrade SG, Armstrong T, Degrave W, Andrade ZA. 2010. In vitro and in vivo experimental models for drug screening and development for Chagas disease. Mem Inst Oswaldo Cruz 105:233–238. https://doi.org/10.1590/S0074-02762010000200022.
24. Ciaravino V, Kropko ML, Rothwell CE, Hovey CA, Theiss JC. 1995. The genotoxicity profile of atorvastatin, a new drug in the treatment of hypercholesterolemia. Mutat Res 343:95–107. https://doi.org/10.1016/0165-1218(95)90076-4.
25. Carvalho RC, Martins WA, Silva TP, Kaiser CR, Bastos MM, Pinheiro LC, Krettli AU, Boechat N. 2016. New pentasubstituted pyrrole hybrid atorvastatin-quinoline derivatives with antiplasmodial activity. Bioorg Med Chem Lett 26:1881–1884. https://doi.org/10.1016/j.bmcl.2016.03.027.
26. Kaiser M, Maes L, Tadoori LP, Spangenberg T, Ioset JR. 2015. Repurposing of the open access malaria box for kinetoplastid diseases identifies novel active scaffolds against trypanosomatids. J Biomol Screen 20:634–645. https://doi.org/10.1177/1087057115569155.
27. Florin-Christensen M, Florin-Christensen J, Garin C, Isola E, Brenner RR, Rasmussen L. 1990. Inhibition of Trypanosoma cruzi growth and sterol biosynthesis by lovastatin. Biochem Biophys Res Commun 166:1441–1445. https://doi.org/10.1016/0006-291X(90)91028-Q.
28. Nzila A, Ma Z, Chibale K. 2011. Drug repositioning in the treatment of malaria and TB. Future Med Chem 3:1413–1426. https://doi.org/10.4155/fmc.11.95.
29. Schmid C, Kuemmerle A, Blum J, Ghabri S, Kande V, Mutombo W, Ilunga M, Lumpungu I, Mutanda S, Nganzobo P, Tete D, Mubwa N, Kisala M, Blesson S, Mordt OV. 2012. In-hospital safety in field conditions of nifurtimox eflornithine combination therapy (NECT) for T. b. gambiense sleeping sickness. PLoS Negl Trop Dis 6:e1920. https://doi.org/10.1371/journal.pntd.0001920.
30. Alirol E, Schrumpf D, Amici Heradi J, Riedel A, de Patoul C, Quere M, Chappuis F. 2013. Nifurtimox-eflornithine combination therapy for second-stage gambiense human African trypanosomiasis: Médecins sans Frontières experience in the Democratic Republic of the Congo. Clin Infect Dis 56:195–203. https://doi.org/10.1093/cid/cis886.
31. Hotez PJ, Pecoul B, Rijal S, Boehme C, Aksoy S, Malecela M, Tapia-Conyer R, Reeder JC. 2016. Eliminating the neglected tropical diseases: translational science and new technologies. PLoS Negl Trop Dis 10:e0003895. https://doi.org/10.1371/journal.pntd.0003895.
32. Coura JR. 2009. Present situation and new strategies for Chagas disease chemotherapy: a proposal. Mem Inst Oswaldo Cruz 104:549–554. https://doi.org/10.1590/S0074-02762009000400002.

33. Chatelain E. 2016. Chagas disease research and development: is there light at the end of the tunnel? Comput Struct Biotechnol J 15:98–103. https://doi.org/10.1016/j.csbj.2016.12.002.
34. Molina I, Salvador F, Sánchez-Montalvá A. 2016. Update Chagas disease. Enferm Infecc Microbiol Clin 34:132–138. https://doi.org/10.1016/j.eimc.2015.12.008.
35. Torrico F, Gascon J, Ortiz L, Alonso-Vega C, Pinazo MJ, Schijman A, Almeida IC, Alves F, Strub-Wourgaft N, Ribeiro I, E1224 Study Group. 2018. Treatment of adult chronic indeterminate Chagas disease with benznidazole and three E1224 dosing regimens: a proof-of-concept, randomised, placebo-controlled trial. Lancet Infect Dis 18:419–430. https://doi.org/10.1016/S1473-3099(17)30538-8.
36. Timm BL, da Silva PB, Batista MM, da Silva FH, da Silva CF, Tidwell RR, Patrick DA, Jones SK, Bakunov SA, Bakunova SM, Soeiro MDN. 2014. In vitro and in vivo biological effects of novel arylimidamide derivatives against Trypanosoma cruzi. Antimicrob Agents Chemother 58:3720–3726. https://doi.org/10.1128/AAC.02353-14.
37. Batista DGJ, Batista MM, Oliveira GM, Borges P, Lannes-Vieira J, Britto CC, Junqueira A, Lima MM, Romanha AJ, Sales Junior PA, Stephens CE, Boykin DB, Soeiro MNC. 2010. Arylimidamide DB766: a potential chemotherapeutic candidate for Chagas disease treatment. Antimicrob Agents Chemother 54:2940–2952. https://doi.org/10.1128/AAC.01617-09.
38. Simões-Silva MR, De Araújo JS, Oliveira GM, Demarque KC, Peres RB, D'Almeida-Melo I, Batista DGJ, Da Silva CF, Cardoso-Santos C, Da Silva PB, Batista MM, Bahia MT, Soeiro MNC. 2017. Drug repurposing strategy against *Trypanosoma cruzi* infection: in vitro and in vivo assessment of the activity of metronidazole in mono- and combined therapy. Biochem Pharmacol 145:46–53. https://doi.org/10.1016/j.bcp.2017.08.025.
39. De Araújo JS, Da Silva CF, Batista DG, Da Silva PB, Meuser MB, Aiub CA, da Silva MF, Araújo-Lima CF, Banerjee M, Farahat AA, Stephens CE, Kumar A, Boykin DW, Soeiro MN. 2014. *In vitro* and *in vivo* studies of the biological activity of novel arylimidamides against *Trypanosoma cruzi*. Antimicrob Agents Chemother 58:4191–4195. https://doi.org/10.1128/AAC.01403-13.
40. Fivelman QL, Adagu IS, Warhust DC. 2004. Modified fixed-ratio isobologram method for studying in vitro interactions between atovaquone and proguanil or dihydroartemisinin against drug-resistant strains of Plasmodium falciparum. Antimicrob Agents Chemother 48:4097–4102. https://doi.org/10.1128/AAC.48.11.4097-4102.2004.
41. Simões-Silva MR, Nefertiti AS, De Araújo JS, Batista MM, Da Silva PB, Bahia MT, Menna-Barreto RS, Pavão BP, Green J, Farahat AA, Kumar A, Boykin DW, Soeiro MN. 2016. Phenotypic screening in vitro of novel aromatic amidines against *Trypanosoma cruzi*. Antimicrob Agents Chemother 60:4701–4707. https://doi.org/10.1128/AAC.01788-15.
42. Soeiro MN, Mota RA, Batista DG, Pereira MC, Meirelles MN. 2002. *Trypanosoma cruzi* infection impairs the endocytosis of zymosan A by cardiomyocytes. Pathobiology 70:69–75. https://doi.org/10.1159/000067307.
43. Pentassuglia L, Graf M, Lane H, Kuramochi Y, Cote G, Timolati F, Sawyer DB, Zuppinger C, Suter TM. 2009. Inhibition of ErbB2 by receptor tyrosine kinase inhibitors causes myofibrillar structural damage without cell death in adult rat cardiomyocytes. Exp Cell Res 315:1302–1312. https://doi.org/10.1016/j.yexcr.2009.02.001.