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Higher oral efficacy of ravuconazole in self-nanoemulsifying systems in shorter treatment in experimental chagas disease

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ABSTRACT

We investigated the *in vitro* activity and selectivity, and *in vivo* efficacy of ravuconazole (RAV) in self-nanoemulsifying delivery system (SNEDDS) against *Trypanosoma cruzi*. Novel formulations of this poorly soluble C14-α-demethylase inhibitor may improve its efficacy in the experimental treatment. *In vitro* activity was determined in infected cardiomyocytes and efficacy *in vivo* evaluated in terms of parasitological cure induced in Y and Colombian strains of *T. cruzi*-infected mice. *In vitro* RAV-SNEDDS exhibited significantly higher potency of 1.9-fold at the IC_{50} level and 2-fold at IC_{90} level than free-RAV. No difference in activity with Colombian strain was observed *in vitro*. Oral treatment with a daily dose of 20 mg/kg for 30 days resulted in 70% of cure for RAV-SNEDDS versus 40% for free-RAV and 50% for 100 mg/kg benznidazole in acute infection (*T. cruzi* Y strain). Long-term treatment efficacy (40 days) was able to cure 100% of Y strain-infected animals with both RAV preparations. Longer treatment time was also efficient to increase the cure rate with benznidazole (Y and Colombian strains). RAV-SNEDDS shows greater efficacy in a shorter time treatment regimen, it is safe and could be a promising formulation to be evaluated in other pre-clinical models to treat *T. cruzi* and fungi infections.

1. Introduction

Chagas disease affects 6 to 7 million people worldwide (WHO, 2020). Currently available anti-*Trypanosoma cruzi* drugs, benznidazole (BZ) and nifurtimox, have limited efficacy in the chronic phase of the infection, when the majority of patients are diagnosed. Recently, BENEFIT-trial, the first multicenter, randomized, double-blind, placebo-controlled clinical trial evaluated the efficacy and safety of treatment with BZ on patients with chronic Chagas heart disease (Morillo et al., 2015). Although parasite levels were reduced in treated patients, BZ was unable to stop their clinical deterioration in a mean of 5 years follow-up (Morillo et al., 2015). In this context, the development of active new chemical entities, the drug-repurposing strategy and the evaluation of specific treatment regimens to prevent morbidity and mortality remain the main lines of research for the future control of Chagas' disease. Among the strategies to overcome the limitations of available treatments, the development of new pharmaceutical formulations to improve the biopharmaceutical properties of active drugs is a faster strategy to obtain more efficient medicines (Bahia et al., 2014).

Antifungal drug-repositioning as anti-*T. cruzi* agents, in particular C14-α-demethylase (CYP51) inhibitors, have been extensively investigated (Diniz et al., 2010; Guedes et al., 2004; Molina, 2000; Urbina et al, 1998, 2003; Villalta et al., 2013). Ravuconazole (RAV) is one of the most potent CYP51 inhibitors tested *in vitro* (Urbina et al., 2003), although its short terminal half-life in mice (4.5 h) and dogs (8 h) limited *in vivo* efficacy profile in these experimental models (Urbina et al., 2003; Diniz et al., 2010). Despite the promising pre-clinical results obtained with RAV and with its water-soluble prodrug fosravucolazole (E1224) and also with posaconazole, these compounds have induced no sustained anti-*T. cruzi* effect in clinical trials (Urbina et al., 2003; Diniz et al.,

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2010; Molina, 2000; Torrico et al., 2018; Molina et al., 2014). Many authors have been suggested that therapeutic failure may be related to suboptimal drug exposure and inadequate azole levels in tissues (Lepesheva et al., 2018; Molina et al., 2014; Torrico et al., 2018; Urbina, 2015, 2017; Villalta and Rachakonda, 2019). RAV pertained to a third-generation of the azole class of antifungals and is poorly soluble in water (<1 μg/ml) and lipophilic (Lentz et al., 2007; Ueda et al., 2003). Most of CYP51 inhibitors are substrates of multidrug resistance pumps also known as permeability glycoprotein (P-gp), expressed in the gut epithelium, liver hepatocytes, endothelial and renal cells (Sakaeda et al., 2005), which are responsible by the drug efflux and reduced drug bioavailability (Wang et al., 2002; European Medicines Agency, 2018; Brüggemann et al., 2009).

Therefore, self-nanoemulsifying delivery systems (SNEDDS) have a potential to block efflux transporters in the gastro-intestinal tract (GIT) and at the same time provide a pre-dissolved state of drug. SNEDDS is a solution of lipid, surfactant and drug, which have been extensively used to improve oral bioavailability of lipophilic drugs, particularly Class II drugs of Biopharmaceutics Classification System (Neslihan Gursoy and Benita, 2004; Müllertz et al., 2010). SNEDDS form fine oil-in-water nanoemulsions upon contact with aqueous GIT content, which increases surface area for drug absorption, as well as activates lymphatic transport (Anton and Vandamme, 2011; Date et al., 2010; O'Driscoll, 2002; Porter et al., 2008; Pouton and Porter, 2008). This formulation could be used to improve RAV biopharmaceutical profile and also increase efficacy in animal models. In previous study we reported a formulation development of RAV incorporated in SNEDDS (RAV--SNEDDS) as a strategy to enhance oral bioavailability of this C14-α-demethylase inhibitor, as well as formulation' excipient safety for healthy mice (Spósito et al., 2017). Therefore, in the present study we evaluated the *in vitro* anti-*T. cruzi* activity of the RAV-SNEDDS and *in vivo* efficacy in mice infected with *T. cruzi* Y (partially sensitive to BZ) and Colombian (resistant to BZ) strains comparing with free-RAV and with BZ-reference treatment.

2. Materials and methods

2.1. Materials

Ravuconazole ([*R*-(*R**,*R**)]-4-[2-[2-(2,4-difluorophenyl)-2-hydroxy-1-metlyl-3-(1*H*-1,2,4-triazol-1-yl)propyl]-4-thiazolyl]benzonitrile) and Resazurin disodium salt were purchased from Sigma-Aldrich (St Louis, MO, USA). Ethanol was provide by Tedia (Rio de Janeiro, Brazil). Benznidazole (*N*-benzyl-2-nitro-1-imidazolacetamide) was a gift from LAFEPE (Pernambuco, Brazil). Cyclophosphamide (Genuxal®) was acquired from Asta Medica Oncologia (São Paulo, Brazil). Soy lecithin (Epikuron®170 with ~75% of phosphatidylcholine) was a gift from Cargill (GmbH, Hamburg, Germany). Labrasol® was a gift from Gattefossé (Cedex, France) and Miglyol®810N was provided by Sasol GmbH (Bremen, Germany).

2.2. Self-nanoemulsifying delivery system preparation

The ravuconazole-loaded SNEDDS (RAV-SNEDDS) were prepared as previously described (Spósito et al., 2017). Briefly, 10 mg of RAV was dissolved in ethanol (50 μl) and mixed with Labrasol (0.10 ml) under magnetic stirring. Afterwards, this solution was mixed with a solution of Miglyol (0.70 ml) and lecithin (0.15 ml) pre-mixed and dissolved under magnetic stirring at 40 °C. Blank-SNEDDS were prepared in the same way without the drug. Freshly prepared formulations were used throughout the *in vitro* and *in vivo* experiments. After auto-emulsification in water this formulation renders a mean hydrodynamic diameter of 200 nm nanoemulsion droplets with zeta potential of −50 ± 3.5 mV with reproducible physicochemical character in the different batches prepared, as was described previously (Spósito et al., 2017).

2.3. Cell viability studies

Two cell lines were used for determination cytotoxicity for RAV and formulations: H9c2 (ATCC: CRL 1446) derived from rat cardiomyoblasts and HepG2 (ATCC: HB 8065) derived from human hepatocyte cell carcinoma. The H9c2 cell line was also used as host cells for *T. cruzi* infection *in vitro* and was therefore used to assess the trypanocidal activity of SNEDDS formulations. The cells were grown in DMEM supplemented with 10% v/v heat-inactivated fetal bovine serum (FBS), 1% v/v of 2 mM L-glutamine, 10.000 UI/ml penicillin and 10 mg/ml streptomycin in 25 cm^2 tissue culture flasks at 37 °C in 5% CO_2 in air.

Stock solutions of RAV were prepared in dimethyl sulfoxide (DMSO) and kept at −20 °C. For the assays, drug solutions and SNEDDS formulations were further diluted at appropriate concentrations using culture medium. The largest DMSO concentration in the wells did not exceed 0.5% v/v. Cytotoxicity assays were performed in 96-well plates, each well receiving 100 μl of culture medium with 1×10^3 of H9c2 cells/well or 5×10^3 for HepG2 cells/well. After 24 h, the medium was removed and the medium containing free RAV or RAV-SNEDDS at different concentrations (20 nM, 10 nM, 5 nM, 2.5 nM, 1.25 nM, 0.625 nM) was added. Blank-SNEDDS was tested under the same experimental conditions and dilutions as for RAV-SNEDDS. After 72 h of incubation the plates are inspected under an inverted microscope to ensure growth of the controls and sterile conditions and resazurin used as an indicator of *in vitro* proliferation of mammalian culture cells by colorimetric reaction (Borra et al., 2009; Riss et al., 2004). Then, in this assay 1 mM resazurin solution was added to each well and incubated for another 4 h for H9c2 cells and for 8 h for HepG2 cells, respectively. The plates were read with a Biochrom Anthos 2010 microplate spectrophotometer at 570 nm and at 600 nm. The results were presented as the average of two independent experiments carried out in triplicate. The selectivity index (SI) was calculated dividing the cytotoxic concentration for 50% of the H9c2 cells (CC_{50}) by inhibitory concentration for 50% of the amastigote form of *T. cruzi* (IC_{50}).

2.4. In vitro anti-T. cruzi activity

For *in vitro* activity assay was conducted as reported (Diniz et al., 2018). We used stock solutions of 1 μM of RAV and equivalent concentration of SNEDDS-RAV. All subsequent dilutions were prepared in culture medium (DMEM) on the day of the assay. DMSO never exceeded 0.5% v/v in medium and had no deleterious effect on parasite growth. For the analysis of the effect against intracellular parasites (amastigotes), the maximum concentration of RAV in different treatments added to the medium was 2 nM and an eight-point profile with two-fold serial dilutions was used in a 72 h assay. Aliquots of 500 μl of H9c2 cell suspension were seeded into 24-well plates at 1×10^4 cell/well. After 24 h the cells were infected with trypomastigotes of the *T. cruzi*, at 15:1 ratio for Y strain, or 30:1 ratio parasites to host cells for Colombian strain. After 24 h of incubation, the non-adherent parasites were removed by washing with DMEM and the cultures were exposed to free-RAV, RAV-SNEDDS or Blank-SNEDDS. All tissue culture plates were maintained at 37 °C in a 5% CO_2/air mixture. After 72 h, the cultures were fixed with methanol, stained with Giemsa, and examined microscopically to determine the percentage of cells infected in treated and untreated controls. In the assays to determine the anti-*T. cruzi* activity of formulations, the untreated controls showed 51.87 ± 6.44% of infected cells in the experiments carried out with Y strain and 58.54 ± 5.96% of infection in those performed with Colombian strain. IC_{50} and IC_{90} values were calculated using *CalcuSyn* software (Biosoft, United Kingdom) (Diniz et al., 2018). All experiments were run in duplicate and the results are given as mean ± standard deviation of two independent experiments.

2.5. In vivo anti-T. cruzi assay

Female Swiss mice (20–24 g) from the Animal Facility at Federal University of Ouro Preto (UFOP), Minas Gerais State, Brazil, were maintained on a diet of commercial food and water available ad libitum under 12 h day/night cycles, temperature 22 ± 2 °C under environmental conditions. The procedures and experimental conditions were in accordance with COBEA (Brazilian School of Animal Experimentation) guidelines for the use of animals in scientific research and approved by the Ethics Committee in Animal Research at UFOP (number 2012/70). Mice were inoculated intraperitoneally with 5000 blood-stage trypomastigotes of the Y (DTU II) or Colombina (DTU I) *T. cruzi* strain. Animals were randomly divided in groups (n = 6 of uninfected mice and n = 8 to 10 of infected groups). Animals we daily treated by oral gavage with BZ, free-RAV, RAV-SEDDS (at equivalent concentrations of free-RAV) or Blank-SEDDS. Additionally, a group of infected animals, but receiving no treatment was used as a control in each experiment. Treatments began at day 4 and 6 post-infection for Y and Colombina infection, respectively. Free-RAV and BZ were orally administrated in suspensions containing carboxymethyl cellulose and 0.5% w/v of polysorbate 80 (Sigma-Aldrich, St. Louis, USA).

The treatment efficacy was determined following the methodology standardized by Caldas et al. (2008), based on parasitemia detection by fresh blood examination (FBE) before and after cyclophosphamide immunosuppression (CyI) and blood qPCR. For qPCR the blood samples were collected 30 and 180 days after treatment from mice with negative fresh blood examination. Animals that showed negative results in all tests were considered cured.

To determined parasitemia, 5 μl of blood were collected from the mice's tail vein and the number of parasites was estimated as previously described (Brener, 1962). Mice were assessed daily for parasitemia during and up to 30 days after treatment. Mortality was cheeked daily until 30 days after treatment. Animals with no parasitemia reactivation in FBE up to 30 days post-treatment were submitted to immunosuppression with cyclophosphamide (Cy). Cy was diluted in ultrapure water and administered intraperitoneally, using three cycles of daily doses of 50 mg/kg of Cy for four consecutive days with intervals of three days between each cycle. Parasitemia was evaluated during the immunosuppression, as well as for the following 10 days after the end of immunosuppression cycles.

To determined real-time PCR, blood of mice (200 μl) was mixed with 35 μl of sodium citrate solution at 129 mM and used for DNA extraction using the Wizard® Genomic DNA Purification Kit (Promega Corp., Madison, WI), according to manufacturer's instructions. Assays of PCR were performed using a SYBR Green system (Roche Applied Science, Mannheim, Germany) and using the primers for *T. cruzi* DNA amplification were TCZ-F 5′-GCTCTTGCCCACAMGGGTGC-3′, where M = A or C (Invitrogen), and TCZ-R 5′-CCAAGCAGCGGATAGTTCAGG-3′, amplify a 182-bp (Cummings and Tarleton, 2003). Primers for murine TNF-α was used as endogenous control normalizing, TNF-5241 5′-TCCCT CTCATCAGTTCTATGGCCCA-3′ e TNF-5411 5′-CAGCAAGCA TCTATGCACTTAGACCCC-3′, amplify a 170-bp product (Cummings and Tarleton, 2003). Cycles of amplification were carried out in a 7500 Fast Real-Time PCR System (Applied Biosystems) and the program consisted of an initial denaturation at 95 °C for 10 min, followed by 40 cycles of 94 °C for 15 s and 64.3 °C for 1 min, with fluorescence acquisition at 64.3 °C. The amplification was immediately followed by a melt program with initial denaturation at 95 °C for 15 s, cooling to 60 °C for 1 min and gradual temperature increase of 0.3 °C/s to 95 °C.

2.6. Statistical analysis

IC_{50} and IC_{90} and CC_{50} concentration values were calculated using *CalcuSyn* software (Biosoft, United Kingdom). Survival was analyzed by non-parametric Kaplan-Meier log rank using GraphPad Software 6.01 (San Diego, CA). The results were expressed as mean ± standard deviation. Parametric data were analyzed with Student's *t*-test and nonparametric data with the Mann–Whitney test. Values of $p < 0.05$ were considered significant.

3. Results

3.1. In vitro cytotoxicity and activity assays

In order to exclude possible cytotoxicity of Blank-SNEDDS and RAV-SNEDDS themselves, the viability of non-infected host cells was estimated *in vitro* using two cell lines (H9c2 and HepG2) and compared with free-RAV (Fig. 1). RAV had no effects on the cell proliferation up to 5 nM in H9c2, as values of 100% viability were detected (Fig. 1A). Similar results were obtained with Blank-SNEEDS, while for RAV-SNEDDS ≥ 90% viability was measured with RAV concentrations up to 5 nM for both cell lines. A decrease in cell viability can be detected at the highest RAV-SNEDDS concentration tested in H9c2 cells (ca. 80% survival at 20 nM, Fig. 1A). HepG2 cells were more sensitive than H9c2 cells. However, incubation of HepG2 cells with free-RAV, RAV-SNEDDS or Blank-SNEDDS at concentrations up to 5 nM had no significant effects on the cell viability, as ≥ 95% values were detected.

Therefore, we investigated the activity of the free-RAV and RAV-SNEDDS on Y strain infected H9c2 cells with non-toxic concentrations of RAV (0–2.0 nM). Direct microscopic quantification in Giemsa-stained *T. cruzi*-infected cultures after 72 h of incubation showed a concentration-dependent reduction in the number of infected cells with both RAV-SNEDDS and free-RAV (Fig. 2). However, the IC_{50} values detected for RAV-SNEDDS (0.09 ± 0.01 nM) were significantly lower than free-RAV treated cultures (0.17 ± 0.03 nM) ($p < 0.001$). At the IC_{90} levels RAV-SNEDDS (0.94 ± 0.19 nM) and free-RAV (1.83 ± 0.20 nM) also showed significant difference in activity ($p < 0.05$). Blank-SNEDDS activity at IC_{50} level was detected at highest concentration (Fig. 2). However, alterations on H9c2 cell morphology were detected by microscopy at this dose (data not shown). Altogether, the results showed an *in vitro* increase in RAV potency when incorporated in SNEDDS in Y strain infected cells.

The results showed that RAV is a safe drug *in vitro* for host cells at the usual *T. cruzi* growth inhibitory concentrations. Even at more than 20-fold the IC_{90} of RAV against amastigotes *in vitro*, the viability of H9c2 cells was only slightly reduced (Fig. 1A). The experimental data allow calculating selectivity index (SI) at IC_{50} level in amastigote form of *T. cruzi* in our experimental conditions. SI was estimated to be > 117 and > 222 for free-RAV and RAV-SNEDDS, respectively for Y strain. No improvement of IC_{50} for RAV-SNEDDS compared with free-RAV was observed *in vitro* against intracellular amastigote in Colombian infected H9c2 cells and SI was >142 for both formulations. No activity for blank-SNEDDS was observed in these experiments (Fig. 2).

3.2. In vivo tolerability of RAV-SNEDDS

RAV-SNEDDS was well tolerated by healthy and infected animals. No significant differences in weight loss/gain were found among healthy animals treated with RAV-SNEDDS for 30 or 40 days (data not shown). Similar findings were obtained with Y strain-infected animals treated with an equivalent dose of RAV (20 mg/kg/day) for 30 or 40 days (Fig. 3A). No significant difference in bodyweight gain is observed in Colombian strain-infected animals treated with BZ or RAV formulations (Fig. 3B).

3.3. In vivo efficacy of RAV-SNEDDS

To assess whether a SNEDDS can improve the efficacy of RAV *in vivo*, we evaluated the ability of RAV-SNEDDS to induce parasitological cure in animals in the acute phase of infection with Y or Colombian strains. Oral treatment with a daily dose of 15 mg/kg of RAV-SNEDDS for 20 days fails to induce cure with both free-RAV and RAV-SNEDDS,

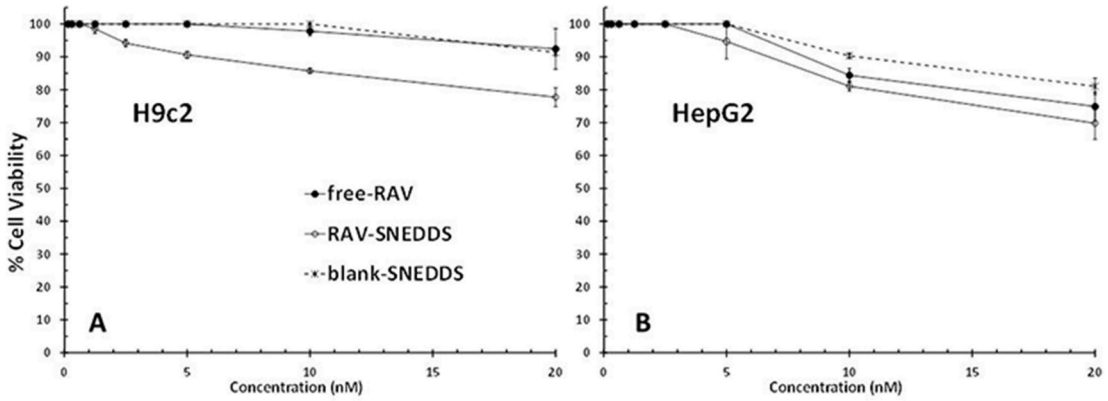

Fig. 1. *In vitro* cytotoxicity toward H9c2 (**A**) and HepG2 (**B**) cells upon incubation for 72 h with free-RAV, RAV-SNEDDS and Blank-SNEDDS. Data is the mean value ± SD.

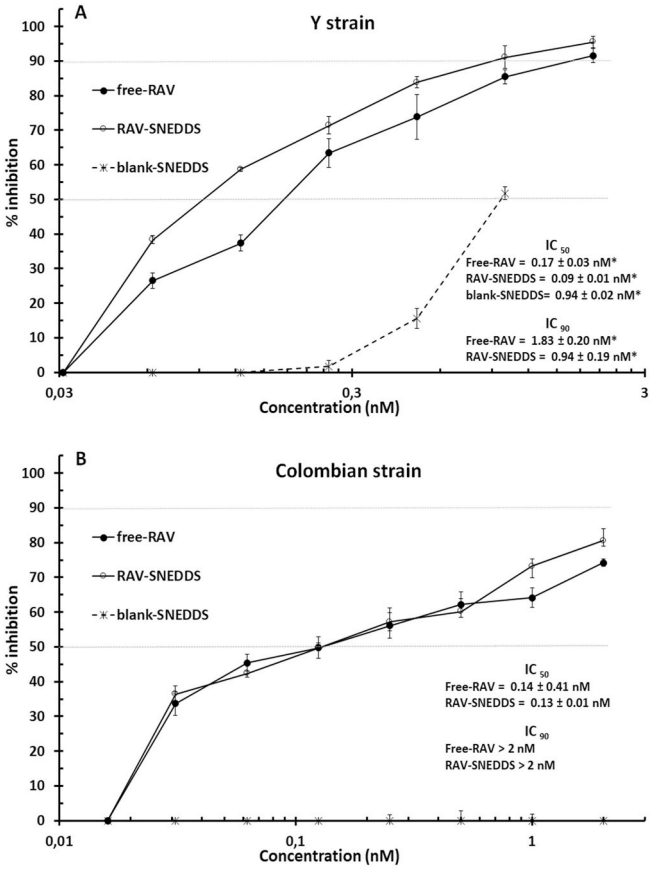

Fig. 2. *In vitro* concentration-dependent effects of free-RAV, RAV-SNEDDS, Blank-SNEDDS on intracellular form of *T. cruzi*-infected cardiomyoblasts (Y or Colombian strain) in a 72 h assay. Inhibition percentage is obtained dividing the number of infected cells in treated culture by the mean value of infected controls culture × 100. Data is the mean value ± SD of results from two independent experiments. *Significant difference between free-RAV and RAV-SNEDDS.

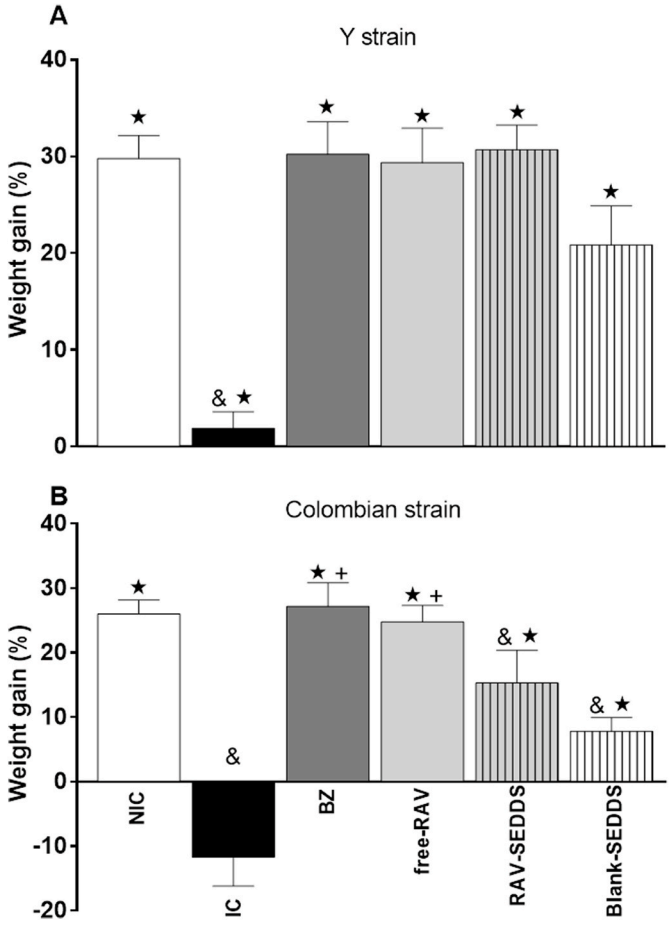

Fig. 3. Weight variation of infected animals treated during 40 consecutive days. Animals were infected with *Trypanosoma cruzi* Y **(A)** or Colombian **(B)** strains and treated with 20 mg/kg of free-RAV, RAV-SNEDDS and 100 mg/kg of BZ. The bodyweight of the infected and untreated control group (IC) was assessed until 14 days after infection for both strains (all animals died before the end of the treatment). ★Significant difference relative to untreated control (IC) ($p < 0.005$); &Significant difference relative to non-infected control ($p < 0.05$).

although fast reduction of parasitemia was observed, with 100% (7/7) mortality for untreated group, 14% (1/7) mortality for free-RAV and no mortality for RAV-SNEDDS (data not shown).

By contrast, daily dose of 20 mg/kg of RAV-SNEDDS for 30 days for Y strain-infected animals resulted in faster suppression of the parasitemia comparable to BZ in the standard protocol and similar to free-RAV (Table 1). All active treatments (free RAV, RAV-SEEDS and BZ) prevented mortality of all treated animals, while 80% of untreated animals succumbed to infection by Y strain of *T. cruzi*. Assessment of definitive clearance of parasites in the animals through a combination of immunosuppression and qPCR of surviving animals indicated that 70%

Table 1
Efficacy and cure control in **Y strain** *T. cruzi*-infected mice treated with ravuconazole formulations for **30 consecutive days** and benznidazole for 20 consecutive days (reference treatment in mice).

Experimental group[a]	Days to achieve parasitemia suppression	Positive test/total number of animals tested[c]		Negative Tests/ total (%)	Mortality (%)[d]
		FBE during and after treatment[b]	PCR		
BZ** (100 mg/kg)	2.1 ± 0.33	2/8	2/6	4/8 (50%)	0/8 (0%)
free-RAV (20 mg/kg)	2.1 ± 0.32	2/10	4/8	4/10 (40%)	0/10 (0%)
RAV-SNEDDS (20 mg/kg)	1.5 ± 0.53	0/10	3/10	7/10 (70%)	0/10 (0%)
Blank-SNEDDS	ND	9/10	1/1	0/10 (0%)	0/10 (0%)
Untreated	ND	–	NP	0/10 (0%)	8/10 (80%)
Non-infected	ND	ND	0/6	6/6 (100%)	0/6 (0%)

ND = not-detected; NP = not-performed.
[a] Swiss mice were inoculated intraperitoneally with 5 × 10[c] bloodstream trypomastigotes of the Y strain of *T. cruzi*. Treatments were given by gavage and started at 4th day after infection for 30 and **20 (BZ) consecutive days.
[b] Positive FBE – positive in FBE during and after treatment (before and after Cy immunossuppression).
[c] Positive PCR = positive result for PCR assay at 1st and 6th month post-treatment. PCR analysis was performed only in FBE negative results.
[d] Mortality was followed up to 30 days after treatment.

of mice infected with the Y strain and treated with RAV-SNEDDS were cured after a 6-month follow-up period, versus 40% for free RAV and 50% for BZ. Interestingly, the blank-SNEDDS showed an anti-*T. cruzi* effect *in vivo*, inducing a reduction of parasitemia compared to untreated mice infected by Y or Colombian strains (data not shown). Blank-SNEDDS was able to prevent death of all animals infected, however no parasitogical cure was observed in this group (Tables 1–3).

Table 2
Efficacy and cure control in **Y strain** *T. cruzi*-infected mice treated with ravuconazole formulations and benznidazole for **40 consecutive days**.

Experimental group[a]	Days to achieve parasitemia suppression	Positive test/total number of animals tested[c]		Negative Tests/ total (%)	Mortality[d] (%)
		FBE during and after treatment[b]	PCR		
BZ (100 mg/kg)	3.6 ± 0.97	0/10	1/10	9/10 (90%)	0/10 (0%)
Free-RAV (20 mg/kg)	1.8 ± 0.42	0/9	0/9	9/9 (100%)	0/9 (0%)
RAV-SNEDDS (20 mg/kg)	1.7 ± 0.48	0/10	0/10	10/10 (100%)	0/10 (0%)
Blank-SNEDDS	ND	9/9	NP	0/9 (0%)	1/9 (11%)
Untreated	ND	–	NP	0/10 (0%)	10/10 (100%)
Non-infected	ND	ND	0/10	10/10 (100%)	0/10 (0%)

ND = not-detected; NP = not-performed.
[a] Swiss mice were inoculated intraperitoneally with 5 × 10[c] bloodstream trypomastigotes of the Y strain of *T. cruzi*. Treatments were given by gavage and started at 4th day after infection for 40 consecutive days.
[b] Positive FBE – positive in FBE during and after treatment (before and after Cy immunossuppression).
[c] Positive PCR = positive result for PCR assay at 1st and 6th month post-treatment. PCR analysis was performed only in FBE negative results.
[d] Mortality was followed up to 30 days after treatment.

Table 3
Efficacy and cure control in **Colombian strain** *T. cruzi*-infected mice treated with ravuconazole formulations and benznidazole for **40 consecutive days**.

Experimental group[a]	Positive test/total number of animals tested[c]		Negative Tests/total (%)	Mortality (%)[d]
	FBE during and after treatment[b]	PCR		
BZ (100 mg/kg)	5/10	5/5	5/10 (50%)	1/10 (10%)
free-RAV (20 mg/kg)	9/10	0/1	1/10 (10%)	1/10 (10%)
RAV-SNEDDS (20 mg/kg)	8/10	0/2	2/10 (20%)	0/10 (0%)
Blank-SNEDDS	10/10	NR	0/10 (0%)	0/10 (0%)
Untreated	–	–	0/10 (0%)	10/10 (100%)
Non-infected	ND	0/6	6/6 (100%)	0/6 (0%)

ND = not-detected; NP = not-performed.
[a] Swiss mice were inoculated intraperitoneally with 5 × 10[c] bloodstream trypomastigotes of the Colombian strain of *T. cruzi*. Treatments were given by gavage and started at 4th day after infection for 40 consecutive days.
[b] Positive FBE – FBE during and after treatment (before and after cyclophosphamide immunossuppression).
[c] Positive PCR = positive result for PCR assay at 1st and 6th month post-treatment. PCR analysis was performed only in FBE negative results.
[d] Mortality was followed up to 30 days after treatment.

Based on these results, we extended the treatment period to 40 days, in order to evaluate the potential of RAV-SNEDDS to induce complete cures in animals infected with Y strain or Colombian strain (Tables 2 and 3). The extended treatment with RAV-SNEDDS led to 100% of cure of animals infected with the Y strain and the same results were achieved in animals treated with free-RAV (Table 2). These results are in agreements with the *in vitro* activity of RAV-SNEDDS, where we observed a significant reduction in the IC_{50} and IC_{90} values, compared to free-RAV. We also investigated if the extended treatment could improve the efficacy of BZ and found that a higher cure rate (90%) was indeed achieved in animals infected with *T. cruzi* Y strain and treated for 40 days (Tables 1 and 2).

Then, we investigated in extended treatment protocol of 40 days the efficacy of RAV-SNEDDS in animals infected by Colombian strain of *T. cruzi*. Daily dose of 20 mg/kg of RAV-SNEDDS for 40 days resulted in fast suppression of the parasitemia with both RAV formulations (Table 3). Treatments with free RAV and RAV-SEEDS prevented the mortality of all treated animals, while 10% of animals died when treated with BZ and Blank-SNEDDS. At the end of the follow-up period 20% of mice treated with RAV-SNEDDS were cured, versus 10% for free-RAV and 50% for BZ (Table 3).

Taken together the results of cure of animals infected with Y or Colombian strains we observe an increase of efficacy with longer treatment protocol for ravuconazole in both formulations (Fig. 4). Therefore, the RAV incorporation in SNEDDS increased the RAV efficacy in shorter treatment of 30 days, with Y strain. Longer treatment of 40 days also increased the efficacy of BZ with both *T. cruzi* strains.

4. Discussion

The lack of efficacy of CYP51 inhibitors in recent clinical trials (Molina et al., 2014; Torrico et al., 2018), despite their anti-*T. cruzi* efficacy demonstrated in animal models, highlighted the need to investigate alternative dosing regimens, possible combined therapies and novel formulations to improve the biopharmaceutical profile and effectiveness of this class of compounds in humans (Urbina, 2015). In this line, SNEDDS formulations are an interesting strategy to improve the efficacy of lipophilic CYP51 inhibitors such as RAV, a poorly water-soluble drug. SNEDDS can be easily prepared and filled in conventional gelatin capsules and represent an alternative to tablets. This formulation can also be administered as an oily anhydrous liquid, dispersible in water, juice or

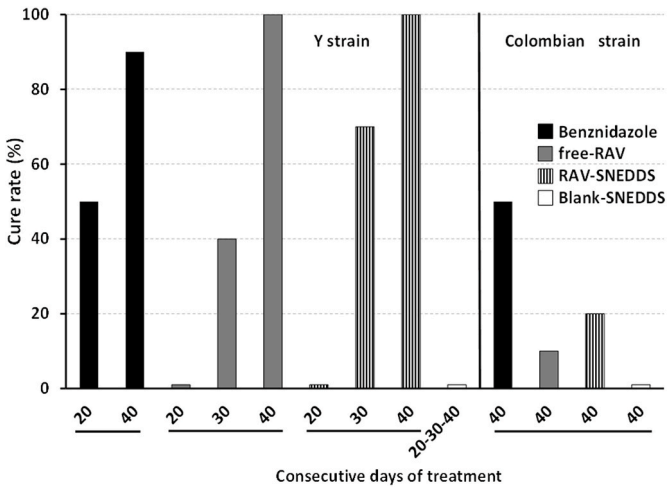

Fig. 4. Comparative efficacy estimated from cure rate (%) of *Trypanosoma cruzi*-infected mice with **Y strain** and **Colombian strain** and treated with free-RAV, RAV-SNEDDS and Blank-SNEDDS for 20 days (15 mg/kg), 30 days (20 mg/kg) and to 40 days (20 mg/kg). BZ was administered for 20 and 40 days at a dose of 100 mg/kg. Cure (%) is based on the criterion that animals with a negative result in all tests were considered as cured: parasite detection in fresh blood examination before and after cyclophosphamide immunosuppression (CyI) and blood qPCR assay at 1st and 6th month post-treatment.

milk, allowing easy dose adjustment and pediatric administration (Mazzeti et al., 2020).

Furthermore, due to its high lipid content, SNEDDS may activate lymphatic transport in the GIT reducing drug first pass-metabolism (Hauss et al., 1998; O'Driscoll, 2002; Date et al., 2010). The surfactants used in the present SNEDDS formulation can alter drug permeability on intestinal cells (Cornaire et al., 2004; Sha et al., 2005). Collectively SNEDDS shows outstanding advantages to improve body exposure to poorly-soluble drug, widely explored commercially (Agrawal et al., 2015).

Considering heart as the main target for parasite infection and inflammation in the Chagas disease cardiomyopathy outcome, we evaluated cytotoxicity of the RAV on rat cardiomyoblasts (H9c2 cells). Furthermore, human hepatocytes are the cells that promote drug metabolization and detoxification in liver. No information was previously available concerning the toxic effects on cell viability of free-RAV, RAV-SNEDDS or Blank-SNEDDS in H9c2 or HepG2 cells. As previously developed and characterized in detail, the RAV-SNEDDS formulation are composed of surfactants to maintain drug in pre-solubilized state (Spósito et al., 2017). Our results showed that RAV-SNEDDS is safe at the usual *T. cruzi* growth inhibitory concentrations *in vitro* for both cells at 72 h incubation, being HepG2 slightly sensitive. The high metabolic activity of these cells and the cell machinery specialized to internalize lipid vesicles (chylomicrons) may be the cause of this difference, particularly considering the composition and physicochemical characteristics of the SNEDDS formulation (Rinninger et al., 1998; Jain et al., 2009). The system of cells in well plates is closed and generally impairs fluid renovation and elimination of xenobiotics, particularly slowly degradable surfactants, as in the case of SEDDS. Even though slight cytotoxicity was observed in our experimental conditions, 23% reduction of viability at the highest concentration for H9c2, the presence of surfactants may damage cell membranes and increase the ravuconazole entry into cells increasing cytotoxicity (RAV-SNEDDS). In HepG2 cells there is no significant difference in the cytotoxicity between the 3 tested formulations, and 30% lost in viability at the highest concentration is considered safe in the majority of cell viability studies. HepG2 cell is metabolically very active, and probably internalize more SNEDDS particles increasing ravuconazole concentration inside cells, probably mediated by membrane destabilization via surfactant.

Even at more than 20-fold the IC_{90} of RAV against amastigotes (Y strain) *in vitro*, the viability of H9c2 cells was only slightly reduced. Selectivity index increases almost 2-fold for RAV-SNEDDS, indicating that SNEDDS formulation increased the safety of RAV for mammalian cells and the selectivity toward the protozoan. BZ SI in *T. cruzi* Y strain infection was reported to be > 36 in NCTC fibroblasts as host cell (Tempone et al., 2017) and 370 in primary cultures of mice cardiac cells (Nefertiti et al., 2017).

In vitro results suggest that the SNEDDS probably acts by improving RAV penetration inside mammalian cells to reach the intracellular amastigotes. Previous work showed that Labrasol surfactant used in RAV-SNEDDS formulation induces enhancement of permeability and dose-dependent cell damage in Caco-2 cells (Cornaire et al., 2004; Sha et al., 2005; Ujhelyi et al., 2012). Thus, plasmatic doses below 5 nM can be considered safe in the *in vivo* environment.

The RAV incorporation in SNEDDS formulation reduces significantly its IC_{50} to 0.09 nM improving the potency of the drug *in vitro* against Y strain intracellular amastigotes of *T. cruzi*. The same was not observed with Colombian strain in the same experimental conditions. Similarly, Moraes et al. (2015) demonstrated variable *in vitro* activity of CYP51 inhibitors upon different clones and strains of *T. cruzi*. Although triazoles have the same molecular target, these different effects may suggest a difference in sequence, number of copies and/or expression of CYP51s in the resistant stocks, leading to different drug affinity (Moraes et al., 2015).

The RAV-SNEDDS was safe to treat infected mice for 20, 30 or 40 days in repeated oral administration. No clinical signs of toxicity were observed during treatment with RAV or RAV-SNEDDS and no weight loss related to toxicity was observed in all protocols studied. The most important finding of the study was that the RAV-SNEDDS formulation produce fast reduction of parasitemia at all doses tested in mice infected with Y and Colombian strains. Particularly, RAV-SNEDDS was significantly more efficacious than free-RAV in suppressing parasitemia, preventing death and inducing radical parasitological cure with a 30 days treatment, 70% and 40%, respectively with Y strain and less pronounced difference, 20% and 10%, with Colombian strain in 40-day schedule (Fig. 4). The treatment with both RAV and RAV-SNEDDS induced complete recovery from bodyweight lost attributed to acute infection compared to untreated mice and healthy mice.

In addition to RAV apparent solubility enhancing effects of SNEDDS (Spósito et al., 2017). Labrasol, the excipient used in SNEDDS, increases the cell membranes fluidity (Koga et al., 2002) potentiate intestinal absorption of different drugs (Eaimtrakarn et al., 2002; Hu et al., 2001; Prasad et al., 2003) and alters the permeability in the intestinal "gap junctions" (Sha et al., 2005). Labrasol also inhibits PgP efflux, thus increasing the drug absorption (Lin et al., 2007). There are no previous studies concerning PgP affinity and/or inhibition by RAV, but this drug is chemically related to fluconazole and itraconazole scaffolds, which are known PgP substrates. Recently, RAV resistance was induced in laboratory (Franco et al., 2020). Thus, SNEDDS formulation would be useful to overcome PgP efflux or the multidrug-resistance phenotypes in trypanosomatids and fungi. Upon administration of RAV-SNEDDS, nanoemulsions of 250 nm in droplet size containing RAV are formed in the GIT providing extensive interfacial area for drug absorption and subsequent release (O'Driscoll, 2002; Wang et al., 2009). We demonstrated that RAV dissolution rate is enhanced in SNEDDS compared with free-RAV suspension (Spósito et al., 2017). It may promote faster absorption by enterocytes compared to free-RAV coarse suspension, which has rate-limited absorption due to its poor water solubility. Furthermore, it was reported recently that a nanometrical formulation increased the efficacy of a sesquiterpene lactone administered by oral route against *T. cruzi*-infected mice, results that corroborate our data (Branquinho et al., 2020; de Mello et al., 2016).

Another important finding was the treatment efficacy improvement in extended 40 days treatment with RAV and RAV-SNEDDS (see Fig. 4). In 40-day treatment (20 mg/kg) effective plasmatic RAV concentrations

were probably achieved by both formulations in mice. The results are also consistent with the finding that an extended 90-days treatment with albaconazole, another CYP51 inhibitor, was safe and able to induced definitive cure in 100% of dogs infected with *T. cruzi* Y strain, while the administration for 60 days was able to cure only 25% of the animals (Guedes et al., 2004). Furthermore, treatment of Y strain-infected mice with a daily dose of 20 mg/kg of RAV for 28 days, followed by a 7-day rest and another 15 days treatment result in 33% of cures, while dosing twice a day resulted in 70% of cures without causing adverse effects in the animals (Urbina et al., 2003). These findings indicate that long-term treatment with azole derivatives can overcome the short half-life of these drugs in mice, without adverse effects to the host. Furthermore, the proposed SEDDS formulation was effective to improve the main limitation of RAV to be overcome in the treatment of Chagas disease, its absorption and pharmacokinetics. Accordingly, the randomized clinical trial to assess the efficacy and safety of posaconazole demonstrated that its administration in 52 chagasic patients for 60 days was safe, only 3 patients had an increase in serum aminotransferase and alkaline phosphatase levels, not having the need to treatment interruption (Molina et al., 2014). Others showed that the treatment of chronic chagasic patients with E-1224 (prodrug of Ravuconazole) for 40–60 days was well tolerated, being a dose-dependent liver enzyme increases was observed (Torrico et al., 2018).

The results of a safety and efficacy clinical trial of fosravuconazole (E-1224) in chronic Chagas disease patients indicated the efficacy of this compound in permanently suppressing parasitemia could be increased from 30 to 90% of patients by extending the treatment from 8 to 12 weeks with 400 mg/week; or by combination therapy with BZ (Torrico et al., 2018). Taken together, these results support the notion that longer treatment regimens may be needed to improve Chagas disease treatment efficacy with azoles derivatives. Our results show that the use of lower doses of RAV-SNEDDS with extended treatment regimens of experimental Chagas disease is an effective therapeutic strategy.

In conclusion, this study indicates that Chagas disease etiological treatment in humans with lipophilic CYP51 inhibitors may require novel pharmaceutical formulations such as SNEDDS and extended therapeutic regimes to provide sustained plasma levels of the drugs. Further research, including a better understanding of the effect of SNEDDS lipids on parasite metabolism and host immunity may help to transfer RAV-SNEDDS to clinical applications as antifungal and anti-*T. cruzi* therapies.

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Author contributions

Conceptualization [PAF Sposito, AL Mazzeti, VCF Mosqueira, MT Bahia], Methodology [PAF Sposito, AL Mazzeti, MT Bahia, KCMP Castro, VCFM], Formal analysis and investigation [PAF Sposito, AL Mazzeti, PF Mendes, KCMP Castro, MTBahia], original draft preparation [PAF Sposito, AL Mazzeti]. Writing-review and editing [AL Mazzeti, VCF Mosqueira, JA Urbina, MT Bahia], funding acquisition, resources, supervision [VCF Mosqueira, MT Bahia]. All authors approve the final version to be published and agree to be accountable for all aspects of the work.

Ethical standards

The procedures and experimental conditions were in accordance with COBEA (Brazilian School of Animal Experimentation) guidelines for the use of animals in scientific research and approved by the Ethics Committee in Animal Research at UFOP (number 2012/70).

Declaration of competing interest

The authors report no conflicts of interest in this work.

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