Characterization and trypanocidal activity of a β-lapachone-containing drug carrier

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Abstract

The treatment of Chagas disease (CD), a neglected parasitic condition caused by Trypanosoma cruzi, is still based on only two drugs, nifurtimox (Nif) and benznidazole (Bz), both of which have limited efficacy in the late chronic phase and induce severe side effects. This scenario justifies the continuous search for alternative drugs, and in this context, the natural naphthoquinone β-lapachone (β-Lap) and its derivatives have demonstrated important trypanocidal activities. Unfortunately, the decrease in trypanocidal activity in the blood, high toxicity to mammalian cells and low water solubility of β-Lap limit its systemic administration and, consequently, clinical applications. For this reason, carriers as drug delivery systems can strategically maximize the therapeutic effects of this drug, overcoming the above-mentioned restrictions. Accordingly, the aim of this study is to investigate the in vitro anti-T. cruzi effects of β-Lap encapsulated in 2-hydroxypropyl-β-cyclodextrin (2HP-β-CD) and its potential toxicity to mammalian cells.

Introduction

Chagas disease (CD), caused by the flagellate protozoan Trypanosoma cruzi, is an endemic illness that affects 21 countries in Latin America. Currently, it is considered by the World Health Organization (WHO) one of the twenty neglected tropical diseases, affecting more than 5 million people worldwide [1–3]. Even 111 years after its discovery, the etiological treatment of CD is still restricted to two nitroheterocycles: benznidazole (Bz) and nifurtimox (Nif) [4]. Their effectiveness varies with the phase of the infection, the dose and period of treatment, and the age and geographical origin of the patient [5]. Additionally, severe adverse reactions and limited efficacy in the chronic phase justify the urgent need for new drugs for CD treatment [6]. For this reason, there is an intensive research effort focused on the search for alternative natural and synthetic new drugs to be used alone or in combination with Bz or with other repurposed drugs [7, 8].
Quinones are present in nature and play an important role in energy production in microorganisms, plants and animals [9]. Naphthoquinones are privileged structures in medicinal chemistry due to their structural and biological characteristics, especially against tumor cells and pathogenic protozoa [10, 11]. The fundamental feature of quinones is their ease of reduction and, therefore, their ability to act as oxidizing or dehydrogenating agents. Their cytotoxicity has been associated with redox cycling, DNA fragmentation, inhibition of human DNA topoisomerase I and II, bioreductive alkylation via the generation of quinonemethides, arylation of the thiol groups of proteins and free radical generation [12–15]. β-Lapachone (β-Lap), a naphthoquinone isolated from the heartwood of trees of the Bignoniaceae family (Tabe-buiasp.), has several bioactive effects, with anti-inflammatory, hepatoprotective, anticancer and antimicrobial properties [16–22]. The anti-T. cruzi activity of β-Lap has been intensively studied by the Docampo group since the 1980s [23, 24]. Unfortunately, the trypanocidal effects of β-Lap are abrogated in the presence of blood and serum, suggesting that it could be inactivated either by reduction in the presence of oxyhemoglobin or by interaction with serum proteins [25]. In addition, the low water solubility of β-Lap limits its systemic administration and clinical use [26], requiring the search for delivery systems [27, 28]. Another approach is the formulation of drugs using colloidal systems, such as liposomes and nanoparticles, to (a) specifically target the affected tissues, (b) improve drug bioavailability and (c) reduce the required dose, thus decreasing toxicity [27, 29, 30]. In this context, cyclodextrins (CDs), a family of cyclic oligosaccharides consisting of a macrocyclic ring of glucose subunits joined by α-1,4 glycosidic bonds, are extensively used in the pharmaceutical industry to obtain nanocomplexes of different drugs. This class of guest molecules can improve drug solubility, stability, and bioavailability [31–33]. Complexation strategies have been successfully explored in studies with Bz to increase its plasma concentration and reduce its in vitro cytotoxicity without impairing biological activity [30, 34–37].

The aim of the present work is to determine the in vitro trypanocidal activity of a new nanostructured CD formulation loaded with β-Lap and its potential toxicity to mammalian cells.

**Materials and methods**

**Chemicals**

Analytical grade solvents were used. Reagents and 2-hydroxypropyl-β-cyclodextrin (2HP-β-CD) were purchased from Sigma-Aldrich or Acros Chemical Co. Ltd., and β-Lapachone was synthesized following a previously reported procedure [38] (Fig 1). The reaction was monitored by thin-layer chromatography carried out using 0.25 mm Merck silica gel plates (60F-254) with UV light as the visualizing agent. The crude product was purified via silica gel (Merck 70–230 mesh) column chromatography using a gradient mixture of hexane and ethyl acetate. Yields refer to purified compounds obtained by chromatographic techniques and confirmed by characterization data obtained from melting points.

**Preparation of the β-Lap:CD complex**

The inclusion complex (β-Lap:CD) of β-LAP and 2-HP-β-CD was prepared by complete dissolution of 2-HP-β-CD (500 mg) in phosphate-buffered saline, pH 7.4 (1 mL) and adding β-LAP (20 mg) with magnetic stirring for 72 h followed by freeze-drying [28].

For all experiments, stock solutions of β-Lap:CD and β-Lap were prepared in PBS and dimethyl sulfoxide, respectively. The final concentration of the solvent in the experiments never exceeded 0.6%, a concentration that is known to not exert toxicity to the parasite or host cells.

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**Competing interests:** The authors have declared that no competing interests exist.
Biological activity

All experiments dealing with animals were performed in accordance with Brazilian Law 11.794/2008 and regulations of the National Council of Animal Experimentation Control under license L038/2018 from the Ethics Committee for Animal Use of the Oswaldo Cruz Institute (CEUA/IOC). Male mice between 3–4 weeks were housed at a maximum of 5 individuals per cage, kept in a specific-pathogen-free (SPF) room at 20 to 22°C under a 12/12 h light/dark cycle at 50 to 60% humidity and provided sterilized water and chow ad libitum [39]. The animals will be euthanized to obtain the parasites on the eighth day after infection to carry out the experiments. The procedure will be carried out by injecting intraperitoneally, in the lower right half of the abdomen, a sedative-analgesic drug associated with anesthetic overdose. After application of the lethal dose of anesthesia we will wait for the loss of pain reflexes (analyzed by clamping the paw) and will be performed cervical dislocation to ensure the animal’s death. Animals infected with T. cruzi that will be used to obtain the parasites will be observed daily for signs that indicate intense suffering. Some of the parameters to be observed are: stay in the corner of the box with the local snout down, prostration and difficulty in eating, weight loss greater than 20% of body weight and goose bumps. When two or more factors change, denoting suffering, or animal will be euthanized.

In vitro activity of β-Lap:CD and β-Lap against the Tulahuen strain of T. cruzi

Culture-derived trypomastigotes of the Tulahuen strain (DTU IV) expressing the Escherichia coli β-galactosidase gene [40] were used to infect L929 mouse fibroblasts (MOI 10:1). After 2 h of interaction, the cultures were washed and maintained for 48 h to establish infection, and then β-Lap:CD or β-Lap was added and incubated for 96 h at 37°C. After this period, 50 μL of chlorophenol red glycoside (500 μM) (CPRG, Sigma-Aldrich) was added to 0.5% Nonidet P40 solution (Sigma-Aldrich), and the plates were incubated for another 18 h. Next, the absorbance
was measured at 570 nm, and the results were expressed as the percent inhibition of infection or *T. cruzi* growth inhibition. The standard drug **Bz** was used as a control [41].

**In vitro activity of β-Lap:CD and β-Lap against the Y strain of T. cruzi**

Evaluation of the **β-Lap:CD** and **β-Lap** activities against intracellular amastigote forms (Y strain) was performed using primary cultures of mouse embryo heart muscle cells (HMCs) as host cells. Briefly, hearts of 18-day-old mouse embryos were fragmented and dissociated with trypsin and collagenase in PBS. Thereafter, isolated cells were cultivated in Dulbecco's modified Eagle medium (DMEM) containing 7% fetal bovine serum (FBS; Cultilab, São Paulo, Brazil), 2.5 mM CaCl₂, 1 mM L-glutamine (Sigma), 2% chicken embryo extract and 1% penicillin/streptomycin solution (Life Technologies, São Paulo, Brazil) and then plated onto gelatin-coated glass coverslips maintained at 37°C in a 5% CO₂ atmosphere [42]. HMCs were infected with bloodstream trypomastigotes (MOI 10:1), and after 24 h, the cultures were washed to remove the noninternalized parasites. Next, **β-Lap:CD** or **β-Lap** was added (2 to 0.7 μM) for 24 h at 37°C. The cultures were fixed and stained with Diff-Quick (Laborclin) and examined by light microscopy to determine the percent infected cells and infection index (II), which corresponds to the number of parasites/100 cells [43]. The results are also expressed using the IC₅₀/24 h, which corresponds to the concentration that produces a 50% decrease in the II [39].

Bloodstream trypomastigotes (Y strain; DTU II) were obtained from the blood of infected Swiss-Webster mice at the peak of parasitemia by heart puncture and isolated by differential centrifugation and resuspended in DME supplemented with 10% fetal calf serum medium [44]. The parasites (5×10⁶ cells/mL) were incubated with **β-Lap:CD** or **β-Lap** for 24 h in two experimental conditions: at 37°C in the absence of blood or at 4°C in the presence of 5% benznidazole, the standard drug, which was used as positive control [11, 43].

Epimastigote forms (Y strain) were maintained axenically at 28°C with weekly transfers in LIT medium and were harvested during the exponential phase of growth. The parasites were incubated with **β-Lap:CD** or **β-Lap** for 24 h at 28°C. For both trypomastigotes and epimastigotes, cell counts were performed in a Neubauer chamber, and the activity was expressed as the IC₅₀/24 h, corresponding to the concentration that led to 50% lysis of the parasites [11].

**In vitro toxicity of β-Lap:CD and β-Lap to mammalian cells**

Cytotoxicity assays were performed using two models, the mouse fibroblast cell line L929, obtained from the American Type Culture Collection (Manassas, VA) (using RPMI-1640 medium, pH 7.2 plus 10% foetal bovine serum and 2 mM L-glutamine) and HMCs using DMEMS. In both cases, 5 × 10⁴ cells in 200 μL of the appropriate culture medium were added to each well of a 96-well microtiter plate and incubated with **β-Lap:CD** or **β-Lap** for 24 h or 96 h at 37°C. Afterwards, PrestoBlue (Invitrogen) was added at a 1:10 ratio in medium, and the microplates were incubated for 5 h. The absorbance was measured at 570 and 600 nm using a spectrophotometer, as recommended by the manufacturer. The results are expressed as the difference in the percent reduction between treated and untreated cells, with the LC₅₀ being the concentration that leads to damage of 50% of the mammalian cells. The selectivity index (SI) was calculated by the ratio between the LC₅₀ and IC₅₀, the latter of which was the concentration that led to 50% lysis/proliferation inhibition of the parasites [45].

**Transmission electron microscopy analysis**

Epimastigotes (Y strain, 5 × 10⁶ cells/mL) were treated for 24 h with **β-Lap:CD** or **β-Lap** at the concentrations corresponding to their IC₅₀/24 h values. The parasites were fixed with 2.5%
glutaraldehyde in 0.1 M Na-cacodylate buffer (pH 7.2) for 40 min at 25°C and post fixed for 20 min at 25°C with 1% OsO₄, 0.8% potassium ferricyanide and 2.5 mM CaCl₂ in buffer. The samples were dehydrated in an ascending acetone series and embedded in Polybed 812 resin. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a JEOL JEM1011 transmission electron microscope (Tokyo, Japan) (Technological Platform of Electronic Microscopy at the Institute of Oswaldo Cruz) [39].

Statistical analysis
Data are expressed as the arithmetic means ± SD of at least three independent experiments. All statistical tests were performed using the Mann-Whitney t test and ANOVA in IBM SPSS Statistics 22.0 software (IBM Corporation, Armonk, New York, USA), p value ≤ 0.05 was considered significant.

Results
First, following our well-established flow chart flux [41], the activity against the intracellular forms of the Tulahuen strain (DTU IV) was performed using the L929-infected cell line. Infected cultures were treated with β-Lap:CD or β-Lap (0.15–10 μM) for 96 h and analyzed by colorimetric analysis. As presented in Table 1, encapsulation increased trypanocidal activity (β-Lap:CD IC₅₀/96h = 0.60 ± 0.05 μM) compared to free naphthoquinone (β-Lap IC₅₀/96h = 2.21 ± 1.25 μM). Although the toxicity of β-Lap:CD to L929 was approximately two times higher than that of β-Lap, leading to SI values of 6.6 and 3.5, respectively, the formulation presented higher selectivity.

To expand the analysis to other T. cruzi strains and mammalian host cells, β-Lap:CD and β-Lap were screened against intracellular forms of the Y strain (DTU II) using HMCs as host cells. To determine the concentrations to be used in the trypanocidal assay, first, the toxicity to the host cell was determined. Since the LC₅₀/24 h values for HMCs were 10.88 ± 4.19 and 6.03 ± 1.40 μM for β-Lap:CD and β-Lap, respectively, the range of nontoxic concentrations for subsequent assays were established as 0.7 to 2 μM.

Light microscopy illustrated the inhibition of HMC infection by the Y strain of T. cruzi after a 24 h of treatment with 1 μM β-Lap:CD or β-Lap (Fig 2A). Quantification of the infected and treated cultures showed dose-dependent inhibition of the infection by both β-Lap:CD and β-Lap (Fig 2B); a similar range of inhibition (20–60%) was observed at concentrations between 0.7 and 2 μM (Fig 2B).

In sequence, the activities of β-Lap:CD and β-Lap on bloodstream trypomastigotes of the Y strain were evaluated. Two experimental conditions were used: in DMES in the absence and presence of blood. Without blood, no difference in trypanocidal activity between β-Lap:CD (IC₅₀/24h = 5.85 ± 0.57 μM) and β-Lap (IC₅₀/24h = 6.13 ± 0.65 μM) was observed (Table 2).

Table 1. Effects of β-Lap:CD and β-Lap on the intracellular forms of T. cruzi (Tulahuen strain) and toxicity to L929 fibroblasts.

|          | IC₅₀/96 h(μM) | LC₅₀/96 h (μM) | SI   |
|----------|--------------|----------------|------|
| β-Lap:CD | 0.60 ± 0.05* | 3.94 ± 0.02     | 6.6b |
| β-Lap    | 2.21 ± 1.25  | 7.76 ± 0.02     | 3.5  |
| Bz       | 2.16 ± 0.98  | 190.6 ± 13.4*   | 88.2 |

*Mean ± SD of at least three independent experiments;  
*b selectivity index (SI) = LC₅₀/IC₅₀;  
*c Simões-Silva et al. 2017 [46].

https://doi.org/10.1371/journal.pone.0246811.t001
The presence of 5% blood and a reduction of the temperature to 4˚C led to a decrease in trypa-nocidal activity in both cases; however, this decrease was 2-fold higher for β-Lap:CD.

In order to investigate whether the encapsulation of β-Lap alters the way in which this naphthoquinone acts on the parasite ultrastructure, epimastigotes of T. cruzi (Y strain) were treated with β-Lap:CD and β-Lap at concentrations corresponding to their IC\textsubscript{50}/24 h values of 11.7 ± 1.6 and 10.2 ± 2.2 μM, respectively. After incubation for 24 h, the materials were processed for transmission electron microscopy and analyzed. In both cases, the most frequent alterations were mitochondrial swelling, disorganization of reservosomes and blebbing in the plasma and flagellum membranes. Epimastigotes treated with β-Lap:CD showed some other alterations, such as large kinetoplasts with altered kDNA compacting patterns, concentric membrane structures involving lipid bodies and reservosomes with the characteristic morphology of autophagosome annal formation of inner vesicles (Fig 3).

**Discussion**

The focus of the present study was to assess the in vitro trypanocidal activity of a new nano-structured CD formulation loaded with β-Lap. In recent years, this quinone has attracted considerable attention, particularly in cancer research [48–50]. The trypanocidal activity of β-Lap and its derivatives has also been extensively demonstrated in vitro and in vivo [51, 52]. However, low water solubility [26] and inactivation of β-Lap in the presence of blood and serum limit its systemic administration and clinical applications for the treatment of CD [13, 25]. It has been hypothesized that encapsulation of this drug could solve these problems, since the use of cyclodextrin in nanocomplexes with Bz was well received, leading to improved solubility and decreased in vitro cytotoxicity [30, 34].

Recently, our group described for the first time the inclusion of β-Lap in cyclodextrin and its activity against T. cruzi, observing that complexation enhanced the SI compared to the free form of this drug [37]. In the present work, aiming to deepen the study of β-Lap complexes, free and encapsulated forms of the quinone were investigated on the three evolutionary forms of the parasite with emphasis on the forms relevant to mammalian infection (amastigotes and trypomastigotes) [2]. Additionally, to expand this study, two different strains of T. cruzi were

**Table 2. Effects of β-Lap:CD and β-Lap on the trypomastigote forms of T. cruzi (Y strain) under two experimental conditions.**

| Compound   | IC\textsubscript{50} (μM) |
|------------|--------------------------|
|            | 0 % blood               | 5 % blood               |
| β-Lap:CD   | 6.13 ± 0.65\textsuperscript{a} | 404.98 ± 24.70          |
| β-Lap      | 5.85 ± 0.57             | 198.85 ± 18.55          |
| Bz         | 8.81 ± 1.08             | 103.60 ± 0.60\textsuperscript{b} |
| CD         | >1000                    |                         |

\textsuperscript{a} Mean ± SD of at least three independent experiments;  
\textsuperscript{b} Da Silva Jr et al. 2008 [47].

https://doi.org/10.1371/journal.pone.0246811.t002
Fig 3. Ultrastructural alterations in *T. cruzi* epimastigotes treated with 10 μM β-Lap:CD or β-Lap. (a) Untreated parasites showing the typical elongated body, with normal morphology of the mitochondrion (M), nucleus (N), flagellum (F), reservosomes (R) and kinetoplast (K). (b-d) β-Lap:CD; (e-h) β-Lap:CD and β-Lap generated similar phenotypic changes, with swelling of the mitochondria (asterisks), disorganization of reservosomes (black star) and blebs in the plasma membrane and flagellum (thick black arrows), presence of large kinetoplasts (K) with an altered...
The encapsulation of $\beta$-Lap improved significantly the activity of the quinone against intracellular amastigotes of the Tulahuen strain in L929 fibroblasts by 3.7 times ($p = 0.028$); however, the difference in the SI value was less noted because the complex was more toxic to the mouse fibroblast lineage than the quinone itself. On the other hand, for the Y strain, no significant differences were observed in the activity of $\beta$-Lap:CD over $\beta$-Lap against amastigotes interiorized in cardiac cells, showing that the encapsulation did not interfere with the trypanocidal activity of the quinone. The distinct behavior or the Tulahuen and Y strains could be due to differences in the standardized protocols employed, and we also cannot discard the differences in susceptibility between both strains.

The encapsulation of $\beta$-Lap did not interfere with the quinone activity against bloodstream trypomastigotes (Y strain) in our standard conditions [43], i.e., DMES medium at 37˚C, with $IC_{50}$ values of 5.85 ± 0.57 and 6.13 ± 0.65 μM for $\beta$-Lap:CD and $\beta$-Lap, respectively. Blood addition (5%) and temperature reduction (from 37˚C to 4˚C) led to a substantial decrease in trypanocidal activity, as expected; however, this decrease was 34-fold higher for the cyclodextrin complex (66X) than for $\beta$-Lap. In view of these results, it is hypothesized that the cyclodextrin formulation could interact with cholesterol and other blood lipids [55–57] and form an insoluble complex [58] that is responsible for the decrease in activity of the encapsulated form of $\beta$-Lap.

Ultrastructural analysis of the treated epimastigotes indicates that the mechanism of action of $\beta$-Lap is preserved with encapsulation, since the phenotypic changes observed with $\beta$-Lap:CD treatment are similar to those induced by $\beta$-Lap, as shown in the present work and in the literature [59, 60]. In this context, studies on $\beta$-Lap encapsulation should be continued, aiming to reverse problems related to the bioavailability of this drug. The results obtained with the Tulahuen strain encourage the development of nanostructures loaded with $\beta$-Lap, since the interaction of new formulations with cholesterol could improve drug stability, reduce the binding to plasma proteins and avoid precipitation [61–63].

**Supporting information**

S1 Graphical abstract.

(JPG)

**Acknowledgments**

The authors thank Dr. Solange Lisboa de Castro for critical reading of the manuscript. The authors thank the Technological Platform of Electron Microscopy at Instituto Oswaldo Cruz.

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References

1. World Health Organization, Chagas disease. 2020; Available from: https://www.who.int/health-topics/chagas-disease#tab=tab_1.

2. Rassi A Jr, Rassi A, Marin-Neto JA. Chagas disease. Lancet. 2010; 375(9723):1388–1402. https://doi.org/10.1016/S0140-6736(10)60061-X PMID: 20399979

3. World Health Organization Chagas disease in Latin America: an epidemiological update based on 2010 estimates. Week Epidemiol Rec. 2015; 90:33–43.

4. Coura JR, de Castro SL. A critical review on Chagas disease chemotherapy. Mem Inst Oswaldo Cruz. 2002; 97(1):3–24. https://doi.org/10.1590/s0074-02762002000100001 PMID: 11992141

5. Coura JR, Borges-Pereira J. Chagas disease. What is known and what should be improved: a systemic review. Rev Soc Bras Med Trop. 2012; 45(3):286–296. https://doi.org/10.1590/s0037-86822012000300002 PMID: 22760123

6. Salomão K, Menna-Barreto RF, de Castro SL. Stairway to Heaven or Hell? Perspectives and Limitations of Chagas Disease Chemotherapy. Current Topics in Medicinal Chemistry. 2016; 16(20):2266–2289. https://doi.org/10.2174/156802661666616041325049 PMID: 27072716

7. Salomão K and De Castro S.L. Recent advances in drug development for Chagas disease: two magic words, combination and repositioning. In: Different Aspects on Chemotherapy of Trypanosomatids. Leon L. and Torres-Santos E. C. (Eds.), Nova Science Publishers, NY, ISBN. 2017; 978-1-5361-850-7, pp. 181–226.

8. Ribeiro V, Dias N, Paiva T, et al. Current trends in the pharmacological management of Chagas disease. Int J Parasitol Drugs Drug Resist. 2020; 12:7–17. https://doi.org/10.1016/j.ijpddr.2019.11.004 PMID: 31862616

9. Powis G. Free radical formation by antitumor quinones. Free Radic Biol Med. 1989; 6(1):63–101. https://doi.org/10.1016/0891-5849(89)90162-7 PMID: 2492250

10. Costantino L, Barlocco D. Privileged structures as leads in medicinal chemistry. Curr Med Chem. 2006; 13(1):65–85. https://doi.org/10.2174/092986706666616041325049 PMID: 16457640

11. Salomão K, De Santana NA, Molina MT, De Castro SL, Menna-Barreto RF. Trypanosoma cruzi mitochondrial swelling and membrane potential collapse as primary evidence of the mode of action of naphthoquinone analogues. BMC Microbiol. 2013; 13:196. https://doi.org/10.1186/1471-2180-13-196 PMID: 24004461

12. Menna-Barreto RF, Corrêa JR, Pinto AV, Soares MJ, de Castro SL. Mitochondrial disruption and DNA fragmentation in Trypanosoma cruzi induced by naphthomidaazoles synthesized from beta-lapachone. Parasitol Res. 2007; 101(4):895–905. https://doi.org/10.1007/s00438-007-0556-1 PMID: 17546464

13. Pinto AV, de Castro SL. The trypanocidal activity of naphthoquinones: a review. Molecules. 2009; 14(11):4570–4590. https://doi.org/10.3390/molecules14114570 PMID: 19924086

14. Ferraz da Costa DC, Pereira Rangel L, Martins-Dinis MMDDC, Ferretti GDDS, Ferreira VF, Silva JL. Anticancer Potential of Resveratrol, ß-Lapachone and Their Analogues. Molecules. 2020; 25(4):893. https://doi.org/10.3390/molecules25040893 PMID: 32085381

15. Pereyra CE, Dantas RF, Ferreira SB, Gomes LP, Silva FP Jr. The diverse mechanisms and anticancer potential of naphthoquinones. Cancer Cell Int. 2019; 19:207. https://doi.org/10.1186/s12935-019-0925-8 PMID: 31388334
16. Carvalho LH, Rocha EM, Raslan DS, Oliveira AB, Krettli AU. In vitro activity of natural and synthetic naphthoquinones against erythrocytic stages of Plasmodium falciparum. Braz J Med Biol Res. 1988; 21(3):485–487. PMID: 3067810

17. Corrêa G, Viela R, Menna-Barreto RF, Midlej V, Benchimol M. Cell death induction in Giardia lamblia: effect of beta-lapachone and starvation. Parasitol Int. 2009; 58(4):424–437. https://doi.org/10.1016/j.parint.2009.08.006 PMID: 19703583

18. Guimarães TT, Pinto Mdo C, Lanza JS, Melo MN, do Monte-Neto RL, de Melo IM, et al. Potent naphthoquinones against antimony-sensitive and -resistant Leishmania parasites: synthesis of novel α- and nor-α-naphtho-1,2,3-triazoles by copper-catalyzed azide-alkyne cycloaddition. Eur J Med Chem. 2013; 63:532–530. https://doi.org/10.1016/j.ejmech.2013.02.038 PMID: 23535320

19. Moraes DC, Curvelo JAR, Anjos CA, et al. β-lapachone and α-nor-lapachone modulate Candida albicans viability and virulence factors. J Mycol Med. 2018; 28(2):314–319. https://doi.org/10.1016/j.jmycmed.2018.03.004 PMID: 29598974

20. Futuro DO, Ferreira PG, Nicoletti CD, et al. The Antifungal Activity of Naphthoquinones: An Integrative Review. An Acad Bras Cienc. 2018; 90(Suppl 2):1187–1214. https://doi.org/10.1590/0003-4983-20180071 PMCID: 29873671

21. Aminin D, Polonik S. 1,4-Naphthoquinones: Some Biological Properties and Application. Chem Pharm Bull (Tokyo). 2020; 68(1):46–57. https://doi.org/10.1248/cpb.c19-00911 PMID: 31902901

22. Ying HZ, Yu CH, Chen HK, et al. Quinonoids: Therapeutic Potential for Lung Cancer Treatment. Biomed Res Int. 2020; 2020:2460565. https://doi.org/10.1155/2020/2460565 PMID: 32337232

23. Docampo R, Cruz FS, Boveris A, Muniz RP, Esquivel DM. Lipid peroxidation and the generation of free radicals, superoxide anion, and hydrogen peroxide in β-lapachone-treated Trypanosoma cruzi epimastigotes. Arch Biochem Biophys. 1978; 186(2):292–297. https://doi.org/10.1016/0003-9861(78)90438-1 PMID: 205176

24. Pinto AV, Ferreira VF, Capella RS, Gilbert B, Pinto MC, da Silva JS. Activity of some naphthoquinones on blood stream forms of Trypanosoma cruzi. Trans R Soc Trop Med Hyg. 1987; 81(4):609–610. https://doi.org/10.1016/0035-9203(87)90427-5 PMID: 3127962

25. Lopes JN, Cruz FS, Docampo R, et al. In vitro and in vivo evaluation of the toxicity of 1,4-naphthoquione and 1,2-naphthoquinone derivatives against Trypanosoma cruzi. Ann Trop Med Parasitol. 1978; 72(6):523–531. https://doi.org/10.1080/00034983.1978.11719356 PMID: 367298

26. Nasongkla N, Wiedmann AF, Bruening A, et al. Enhancement of solubility and bioavailability of beta-lapachone using cyclodextrin inclusion complexes. Pharm Res. 2003; 20(10):1626–1633. https://doi.org/10.1023/a:1026143519395 PMID: 14620518

27. Ferreira VF, Nicoletti CD, Ferreira PG, Futuro DO, da Silva FC. Strategies for Increasing the Solubility and Bioavailability of Anticancer Compounds: β-Lapachone and Other Naphthoquinones. Curr Pharm Des. 2016; 22(39):5899–5914. https://doi.org/10.2174/1381612822666160611012532 PMID: 27291398

28. Nicoletti C. D., Queiroz M. de S. H., Lima C. G. de S., Silva F. de C. da, Futuro D. O., Ferreira V. F. An improved method for the preparation of β-lapachone:2-hydroxypropyl-β-cyclodextrin inclusion complexes. J Drug DelivSci Techn. 2020a; 58, 101777–101781. https://doi.org/10.1016/j.jddst.2020.101777

29. Scalise ML, Arrúa EC, Rial MS, Esteva MI, Salomon CJ, Fichera LE. Promising Efficacy of Benznidazole Nanoparticles in Acute Trypanosoma cruzi Murine Model: In-Vitro and In-Vivo Studies. Am J Trop Med Hyg. 2016; 95(2):388–393. https://doi.org/10.4269/ajtmh.15-0889 PMID: 27246447

30. Vinuesa T, Herráez R, Oliver L, et al. Benznidazole Nanofluids: A Chance to Improve Therapeutics for Chagas Disease. Am J Trop Med Hyg. 2017; 97(5):1469–1476. https://doi.org/10.4269/ajtmh.17-0044 PMID: 29016287

31. Lach JL, Chin TF. Interaction of pharmaceuticals with schardinger dextrins. iii. interactions with mono-halogenated benzoic acids and aminobenzoic acids. J Pharm Sci. 1964; 53:69–73. https://doi.org/10.1002/jps.2600530112 PMID: 14106377

32. Stella VJ, Rajewski RA. Cyclodextrins: their future in drug formulation and delivery. Pharm Res. 1997; 14(5):556–567. https://doi.org/10.1023/a:1012136606249 PMID: 9165524

33. Grillo R, Melo NF, Moraes CM, et al. Hydroxymethyl nitrofurazone: dimethyl-beta-cyclodextrin inclusion complex: a physical-chemistry characterization. J Biol Phys. 2007; 33(5–6):445–453. https://doi.org/10.1007/s10867-008-9054-7 PMID: 19669530

34. Maximiano FP, Costa GH, de Sá Barreto LC, Bahia MT, Cunha-Filho MS. Development of effervescent tablets containing benznidazole complexed with cyclodextrin. J Pharm Pharmacol. 2011; 63(6):786–793. https://doi.org/10.1111/j.2042-7158.2011.01284.x PMID: 21585376

35. Alves-Silva I., Sá-Barreto L. C. L., Lima E. M. and Cunha-Filho M. S. S. Preformulation studies of triclozanole associated with benznidazole and pharmaceutical excipients. Thermochimica Acta, 2014; 575, 29–33. https://doi.org/10.1016/j.tca.2013.10.007.
36. Leonardo D, Bombardiere ME, Salomon CJ. Effects of benzimidazole: cyclodextrin complexes on the drug bioavailability upon oral administration to rats. Int J BiolMacromol. 2013; 62:543–548. https://doi. org/10.1016/j.jibiomac.2013.10.007 PMID: 24120966

37. Nicoletti CD, Faria AFM, de Sá Haddad Queiroz M, et al. Synthesis and biological evaluation of β-lapachone and nor-β-lapachone complexes with 2-hydroxpropyl-β-cyclodextrin as trypanocidal agents. J Biomed Biopharm. 2020b; 52(3):185–197. https://doi.org/10.1007/s10863-020-09826-8 PMID: 32198699

38. Ferreira VF, Ferreira SB, da Silva F de C. Strategies for the synthesis of bioactive pyrannaphthoqui-ones. Org Biomol Chem. 2010; 8(21):4793–4802. https://doi.org/10.1039/c0ob00277a PMID: 20838670

39. Freitas RHCN, Barbosa JMC, Bernardino P, et al. Synthesis and trypanocidal activity of novel pyridinyl-1,3,4-thiadiazole derivatives. Biomed Pharmacother. 2020; 127:111062. https://doi.org/10.1016/j. biopharm.2020.111062 PMID: 32407986

40. Buckner FS, Verlinde CL, La Flamme AC, Van Voorhis WC. Efficient technique for screening drugs for activity against Trypanosoma cruzi using parasites expressing beta-galactosidase. Antimicrob Agents Chemother. 1996; 40(11):2592–2597. https://doi.org/10.1128/AAC.40.11.2592 PMID: 8913471

41. Romanhã AJ, Castro SL, Soeiro MNC, et al. In vitro and in vivo experimental models for drug screening and development for Chagas disease. Mem Inst Oswaldo Cruz. 2010; 105(5):233–238. https://doi.org/10.1590/s0074-02762010000200022 PMID: 20428868

42. Meirelles MN, de Araujo-Jorge TC, Miranda CF, de Souza W, Barbosa HS. Interaction of Trypanosoma cruzi with heart muscle cells: ultrastructural and cytochemical analysis of endocytic vacuole formation and effect upon myogenesis in vitro. Eur J Cell Biol. 1986; 41(2):198–206. PMID: 3093234

43. Salomão K, de Souza EM, Carvalho SA, et al. In vitro and in vivo activities of 1,3,4-thiadiazole-2-arylhy-drazones of megazol against Trypanosoma cruzi. Antimicrob Agents Chemother. 2010; 54(5):2023–2031. https://doi.org/10.1128/AAC.01241-09 PMID: 20231995

44. Meirelles MN, Souto-Pradón T, De Souza W. Participation of cell surface anionic sites in the interaction between Trypanosoma cruzi and macrophages. J Submicrosc Cytol. 1984; 16(3):533–545. PMID: 6381750

45. Jardim GAM, Silva TL, Goulart MOF, et al. Rhodium-catalyzed C-H bond activation for the synthesis of quinoline compounds: Significant Anti-Trypanosoma cruzi activities and electrochemical studies of functionalized quinones. Eur J Med Chem. 2017; 136:406–419. https://doi.org/10.1016/j.ejmech.2017.05.011 PMID: 28521262

46. Simões-Silva MR, De Araújo JS, Oliveira GM, et al. 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. 2017; 145:44–53. https://doi.org/10.1016/j.bcp.2017.08.025 PMID: 28870526

47. da Silva EN Jr, Menna-Barreto RF, Pinto Mdo C, et al. Naphthoquinoidal [1,2,3]-triazole, a new structural moiety active against Trypanosoma cruzi. Eur J Med Chem. 2008; 43(8):1774–1780. https://doi.org/10.1016/j.ejmech.2007.10.015 PMID: 18045742

48. Li CJ, Li YZ, Pinto AV, Pardee AB. Potent inhibition of tumor survival in vivo by beta-lapachone plus taxol: combining drugs imposes different artificial checkpoints. Proc Natl Acad Sci U S A. 1999; 96(23):13369–13374. https://doi.org/10.1073/pnas.96.23.13369 PMID: 10557327

49. Cardoso MFDC, Salomão K, Bombaça AC, da Rocha DR, da Silva FC, Cavaleiro JAS, et al. Synthesis and anti-Trypanosoma cruzi activity of new 3-phenylthio-nor-β-lapachone derivatives. Bioorg Med Chem. 2015; 23(15):4763–4768. https://doi.org/10.1016/j.bmcc.2015.05.039 PMID: 26118399

50. da Silva Júnior EN, Jardim GAM, Jacob C, Dhawa U, Ackermann L, de Castro SL. Synthesis of quiniones with highlighted biological applications: A critical update on the strategies towards bioactive compounds with emphasis on lapachones. Eur J Med Chem. 2019; 179:863–915. https://doi.org/10.1016/ ejmech.2019.06.056 PMID: 31306817

51. Ferreira SB, Salomão K, de Carvalho da Silva F, et al. Synthesis and anti-Trypanosoma cruzi activity of β-lapachone analogues. Eur J Med Chem. 2011; 46(7):3071–3077. https://doi.org/10.1016/j.ejmech.2011.03.012 PMID: 21450374

52. Cascabulho CM, Meuser-Beatista M, Moura KCG, Pinto MDC, Duque TLA, Demarque KC, et al. Anti-parasitic and anti-inflammatory activities of β-lapachone-derived naphthoimidazoles in experimental acute Trypanosoma cruzi infection. Mem Inst Oswaldo Cruz. 2020; 115:e190389. https://doi.org/10.1590/0074-027620190389 PMID: 32074167

53. Filardi LS, Brenner Z. Susceptibility and natural resistance of Trypanosoma cruzi strains to drugs used clinically in Chagas disease. Trans R Soc Trop Med Hyg. 1987; 81(5):755–759. https://doi.org/10.1016/ 0035-9023(87)90020-4 PMID: 3130683

54. Zingales B, Miles MA, Campbell DA, Tibayrenc M, Macedo AM, Teixeira MM, et al. The revised Trypanosoma cruzi subspecific nomenclature: rationale, epidemiological relevance and research
55. Ohtani Y, Irie T, Uekama K, Fukunaga K, Pitha J. Differential effects of alpha-, beta- and gamma-cyclodextrins on human erythrocytes. Eur J Biochem. 1989; 186(1–2):17–22. https://doi.org/10.1111/j.1432-1033.1989.tb15171.x PMID: 2598927

56. Ohvo H, Slotte JP. Cyclodextrin-mediated removal of sterols from monolayers: effects of sterol structure and phospholipids on desorption rate. Biochemistry. 1996; 35(24):8018–8024. https://doi.org/10.1021/bi9528816 PMID: 8672506

57. Szentel, Singhal A, Domokos A, Song B. Cyclodextrins: Assessing the Impact of Cavity Size, Occupancy, and Substitutions on Cytotoxicity and Cholesterol Homeostasis. Molecules. 2018; 23(5):1228. https://doi.org/10.3390/molecules23051228 PMID: 29783784

58. Stella VJ, He Q. Cyclodextrins. Toxicol Pathol. 2008; 36(1):30–42. https://doi.org/10.1177/0192623307310945 PMID: 18337219

59. Docampo R, Lopes JN, Cruz FS, Souza W. Trypanosoma cruzi: ultrastructural and metabolic alterations of epimastigotes by β-lapachone. Exp Parasitol. 1977; 42(1):142–149. https://doi.org/10.1016/0014-4894(77)90071-6 PMID: 324785

60. Dos Anjos DO, Sobral Alves ES, Gonçalves VT, et al. Effects of a novel β-lapachone derivative on Trypanosoma cruzi: Parasite death involving apoptosis, autophagy and necrosis. Int J Parasitol Drugs Drug Resist. 2016; 6(3):207–219. https://doi.org/10.1016/j.ijpddr.2016.10.003 PMID: 27770751

61. Kohli AG, Kierstead PH, Venditto VJ, Walsh CL, Szoka FC. Designer lipids for drug delivery: from heads to tails. J Control Release. 2014; 190:274–287. https://doi.org/10.1016/j.jconrel.2014.04.047 PMID: 24816069

62. Gharib R, Greige-Gerges H, Fourmentin S, Charcosset C, Auezova L. Liposomes incorporating cyclodextrin-drug inclusion complexes: Current state of knowledge. Carbohydr Polym. 2015; 129:175–186. https://doi.org/10.1016/j.carbpol.2015.04.048 PMID: 26050903

63. Eloy JO, Claro de Souza M, Petrilli R, Barcellos JP, Lee RJ, Marchetti JM. Liposomes as carriers of hydrophilic small molecule drugs: strategies to enhance encapsulation and delivery. Colloids Surf B Biointerfaces. 2014; 123:345–363. https://doi.org/10.1016/j.colsurfb.2014.09.029 PMID: 25280609