Rev Soc Bras Med Trop 51(6):849-853, Nov-Dec, 2018
doi: 10.1590/0037-8682-0499-2017

Short Communication

Antiprotozoal action of synthetic cinnamic acid analogs

***Ana Paula de Azevedo dos Santos*[1],[2],[3], *Saara Nery Fialho*[1],[5], *Daniel Sol Sol de Medeiros*[1],[2],[3], *Ana Fidelina Gómez Garay*[2],[3],[4], *Jorge Alfonso Ruiz Diaz*[2],[3],[4], *Maria Celeste Vega Gómez*[4], *Carolina Bioni Garcia Teles*[1],[2],[5],[6] *and Leonardo de Azevedo Calderon*[1],[2],[3]**

[1]. Plataforma de Bioensaios em Malária e Leishmaniose, Fundação Oswaldo Cruz, Porto Velho, RO, Brasil.
[2]. Programa de Pós-graduação em Biologia Experimental, Universidade Federal de Rondônia, Porto Velho, RO, Brasil.
[3]. Centro de Estudos de Biomoléculas Aplicadas à Saúde, Universidade Federal de Rondônia, Porto Velho, RO, Brasil.
[4]. Centro para el Desarrollo de Investigación Científica, Asunción, Paraguay.
[5]. Centro Universitário São Lucas, Porto Velho, RO, Brasil.
[6]. Instituto Nacional de Epidemiologia na Amazônia Ocidental, Porto Velho, RO, Brasil.

Abstract

Introduction: Leishmaniasis, Chagas disease, and malaria cause morbidity globally. The drugs currently used for treatment have limitations. Activity of cinnamic acid analogs against *Leishmania* spp., *Trypanosoma cruzi*, and *Plasmodium falciparum* was evaluated in the interest of identifying new antiprotozoal compounds. **Methods**: *In vitro* effects of analogs against *L. braziliensis*, *L. infantum chagasi*, *T. cruzi*, and *P. falciparum*, and hemolytic and cytotoxic activities on NCTC 929 were determined. **Results**: Three analogs showed leishmanicidal and tripanocidal activity. No antiplasmodial, hemolytic, or cytotoxic activity was observed. **Conclusions**: Antiprotozoal activity of analogs against *L. infantum braziliensis*, *L. infantum chagasi*, and *T. cruzi* was demonstrated.

Keywords: Bioactivity. Cinnamic acid. Leishmaniasis. Synthetic.

The World Health Organization (WHO) lists 17 Neglected Tropical Diseases (NTDs), most of which are caused by protozoa. These diseases cause serious health problems, affecting more than one billion people, most of who live in extreme poverty in tropical and subtropical regions. These diseases are widespread, with an incidence of over 10% in the world population, and yet, their treatments are highly toxic and sometimes ineffective[1], presumably due to lack of attention from the scientific community.

Among these, leishmaniasis is responsible for high morbidity and mortality rates, with an average of 12 million infected people, and about 350 million living in at-risk areas in 98 countries on five continents[1]. During the chemotherapeutic treatment of leishmaniasis, pentavalent antimonials (Sb^{+5}), pentamidines, and amphotericin B are used. However, these treatments have their limitations, including high cost, exclusive venous administration, cardiac alterations, hepatic and renal toxicity, and the emergence of strains resistant to treatment[2].

Trypanosoma cruzi is a flagellate protozoan, responsible for causing Chagas disease. Fourteen million people are infected with *T. cruzi* worldwide, causing approximately 7000 deaths per year, with more than 25 million people living in at-risk areas[1]. Nifurtimox and benznidazole, the currently available treatment options, are relatively toxic, poorly effective in the chronic phase of the disease, and their prolonged administration encourages patient evasion leading to the emergence of resistant strains[3].

In this group of diseases that pose public health challenges, malaria, caused by *Plasmodium falciparum*, stands out, especially in endemic areas such as Africa and Asia, where it is responsible for high rates of morbidity and mortality[1]. The development of resistance of these parasites to major therapeutic compounds, artemisinin and quinine derivatives, makes the search for new bioactive molecules important, especially those studied from a biodiversity perspective[4].

Investing in the discovery of new molecules may have solutions in biodiversity. In this context, plants of the genus *Piper* L. (Piperaceae) are composed of components such as: alkaloids, flavonoids, arylpropanoids, terpenoids, phenylpropanoids, lignans, and cinnamic acid; which are characterized by antifungal, antioxidant, antiplasmodial, and trypanocidal bioactivity[5,6,7]. Among these compounds, cinnamic acid (CinAc) was selected as a model in this study's search for synthetic analogs with trypanocidal and antimalarial action[6,7]. By using this leader molecule we intend to enable the development of more effective drugs with low toxicity, thus presenting a new alternative for treating such diseases.

Corresponding author: Ana Paula de Azevedo dos Santos.
e-mail: paulaazevedo.2011@gmail.com
Received 23 December 2017
Accepted 5 June 2018

Synthetic analogs are derivatives of CinAc, isolated from the plant *P. tuberculatum* Jacq. These compounds **(Figure 1)** were synthesized by Sigma (Sigma-Aldrich).

The analogs were diluted in dimethylsulfoxide-DMSO (Sigma-Aldrich) at concentrations at or below 0.6%. The drugs pentamidine, benznidazole, and artemisinin were used as negative controls (100% death) for *Leishmania*s spp., *T. cruzi*, and *P. falciparum*, respectively.

L. braziliensis (MHOM/BR/75/M2904) and *L. infantum chagasi* (MCAN/ES/92/BNC 83) promastigotes were maintained *in vitro* in RPMI 1640 medium (Sigma-Aldrich) supplemented with 25 mM HEPES (Sigma-Aldrich), 21 mM sodium bicarbonate (Sigma-Aldrich), 11 mM glucose (Sigma-Aldrich), 40 μg/mL of 10% gentamycin (v/v), and 10% previously inactivated fetal bovine serum (FBS) (Sigma-Aldrich). The parasites were kept in a BOD incubator at 26 ºC, and subcultured every five days.

Trypanosoma cruzi epimastigotes, "CL-B5" strain, were grown in liver infusion tryptose culture medium (LIT, Sigma-Aldrich) supplemented with 10% FBS; cultures were kept in a BOD incubator at 24 °C for weekly subculturing.

Chloroquine-resistant and mefloquine-sensitive *P. falciparum* (W2) strain derived from the Indochin III/CDC strain were cultured *in vitro* in human red blood cells with 2% hematocrit, diluted in RPMI 1640 culture medium supplemented with Albumax (Thermo Fisher Scientific). The cultures were maintained in desiccators at 37 °C in a gasogenic mixture containing 5% O_2, 5% CO_2, and 90% N_2.

L. braziliensis and *L. infantum chagasi* promastigotes were plated at 1.5×10^6 parasites/mL in 96-well microplates, incubated at 26 °C for 72 h with varied concentrations (1260-9.8 μM) of the analogs. Subsequently, a 3 mM solution of alamarBlue (Sigma-Aldrich) was added and incubated at 26 °C for 4 h. Absorbance was monitored at 570 and 600 nm using a Synergy H1 (Biotek) multimodal spectrophotometer. Positive controls (parasites with no treatment) and negative controls in pentamidine, at varied concentrations (9-0.14 μM), and DMSO (0.6%) were used in order for the assay to be effective.

T. cruzi epimastigotes were plated at 2.5×10^5 parasites/mL on microplates and incubated at 24 °C for 72 h in serial concentrations of the analogs (1,260-9.8 μM). Subsequently, a solution of 200 μM chlorophenolRed-β-D-galactopyranoside (CPRG) was added followed by incubation at 37 °C for 4 h; absorbance was monitored using a Synergy H1 multimodal spectrophotometer (Biotek) at 570 nm[8]. Parasites incubated with culture medium were considered as the growth control; reference drug benznidazole (100-1.56 μM) was used as a negative control.

P. falciparum cultures (W2) were treated with 5% sorbitol (Sigma-Aldrich), for synchronization in young trophozoites; parasitemia (0.05%) was determined by optic microscopy using a Rapid Panoptic kit (Laborclin, BR). Analogs were added to microplates at varied concentrations (1,260-9.8 μM), and infected red cells (positive control) and varied concentrations (0.17-0.0026 μM) of Artemisinin (negative control) were used; the activity was evaluated for 48 h at 37 °C.

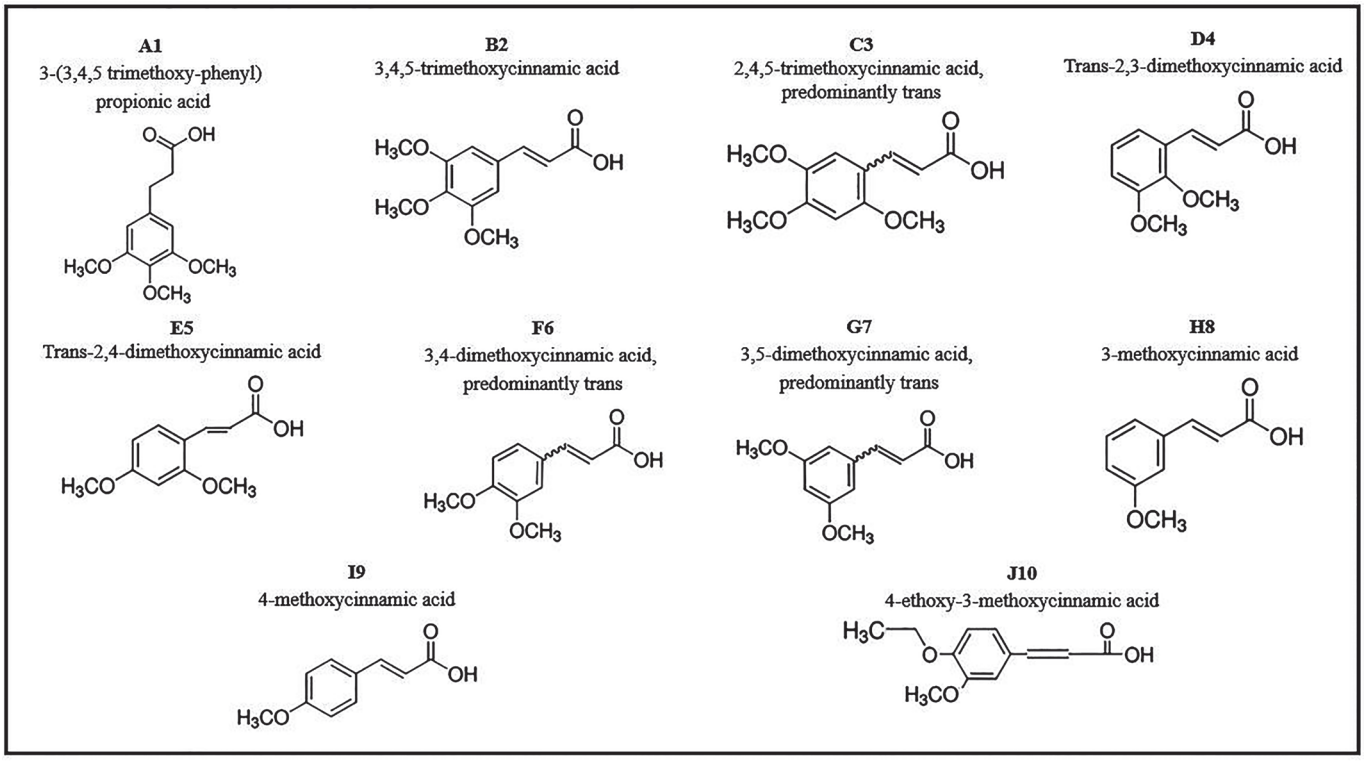

FIGURE 1: Analogous synthetic derivatives of cinnamic acid.

The antimalarial evaluation was performed using SYBR (SYBR Green I, Thermo Fiser). RBCs were washed with 1× PBS and centrifuged at 700 *g* for 10 min. Then, the supernatant was discarded and 100 µL of a SYBR Green I solution (0.001% v/v in lysis buffer) was added; the contents were transferred to microplates containing 100 µL of 1x PBS. The plates were incubated for 30 min at room temperature; fluorescence was obtained at an excitation of 485 nm and an emission of 535 nm[9].

The cytotoxicity was assessed using NCTC 929 fibroblasts in an alamarBlue assay. Cells were plated in microplates with approximately 2.5×10^4 cells/well and incubated with varied concentrations of the analogs (1,260-9.8 µM) for 48 h in an incubator at 37 °C in a humid atmosphere containing 5% CO_2. Subsequently, a 3 mM solution of alamarBlue was added followed by incubated at 37 °C for 4 h. Absorbance was monitored at 570 and 600 nm using a Synergy H1 (Biotek) multimodal spectrophotometer. The 50% cytotoxic concentration for cell growth (CC_{50}) in the presence of the analogs and the control drugs was determined and compared with cells grown only in culture medium. The activity of the molecules tested was estimated by calculating the percentage of viable cells.

The 50% inhibitory concentration of parasite (IC_{50}) and CC_{50} was determined by dose-response curves based on non-linear regression, applying the formula y = A1 + (A2-A1)/(1+10^((LOGx0-x)*p)), with determination of the level of significance at $p < 0.05$. The program Origin (OriginLab Corporation, Northampton, MA 01060, USA) was used. Standard deviation was calculated using MSExcel software. All experiments were performed in triplicates.

The selectivity index (SI) of samples was obtained by calculating the ratio between CC_{50} and IC_{50}. Values > 10 were considered nontoxic, while substances with values below ten were considered toxic[10].

Another way to assess the safety of a drug is to measure its hemolytic activity. Hemolytic assays were performed at varied concentrations (1,260-9.8 µM); 180 µL of erythrocytes (human) were added with 1% hematocrit in microplates with a U-bottom. The plates were incubated for 30 min at 37 °C with agitation after every 5 min. They were then centrifuged for 10 min. The supernatant was analyzed using a microplate spectrophotometer (Biochrom model: Expert plus) at 540 nm. Saponin (0.05%) was used as a positive control for hemolysis.

TABLE 1: Leishmanicidal, trypanocidal, antiplasmodial, and cytotoxic evaluation of CinAc synthetic analogs.

Compounds	IC_{50}(µM) ± SD						
	L. braziliensis[a]	*L. infantum*[b]	*T. cruzi*[c]	*P. falciparum*[d]	SI[e]	SI[f]	SI[g]
A1	> 1,260	> 1,260	> 1,260	> 1,260	NC	NC	NC
B2	> 1,260	> 1,260	> 1,260	> 1,260	NC	NC	NC
C3	> 1,260	> 1,260	> 1,260	> 1,260	NC	NC	NC
D4	> 1,260	> 1,260	> 1,260	> 1,260	NC	NC	NC
E5	340.4 ± 0.93	212.7 ± 0.57	1071.6 ± 1.2	> 1,260	> 3.7	> 5.9	>1.2
F6	> 1,260	> 1,260	> 1,260	> 1,260	NC	NC	NC
G7	> 1.260	642.9 ± 1	667.1 ± 2.9	> 1,260	NC	> 1.9	> 1.9
H8	1018.3 ± 0.8	772.8 ± 0.9	987.2 ± 2.3	> 1,260	> 1.2	> 1.6	> 1.3
I9	248.5 ± 0.8	582 ± 0.8	723 ± 3.3	> 1,260	> 5	> 2.2	>1.7
J10	> 1.260	1110 ± 1.5	1133.9 ± 2.8	> 1,260	NC	> 1.1	> 1.1
PTM	0.76 ± 0.04	1.4 ± 0.26	NT	NT	NT	NT	NT
BZND	NT	NT	9.6 ± 0.9	NT	NT	NT	NT
ARTN	NT	NT	NT	0.02 ± 0.09	NT	NT	NT

IC_{50}: 50% inhibitory concentration/Inhibition of 50% of parasite; Mean **± SD:** results correspond to the average of three trials for each sample and standard deviation. [a]*L. braziliensis* (MHOM/BR/75/M2904); [b]*L. infantum chagasi* (MCAN/ES/92/BNC 83); [c]*T. cruzi* (CL-B5 clone); [d]*P. falciparum* (Indochina III/CDC); **SI:** Selectivity Index, **SI[e]:** *L. braziliensis;* **SI[f]:** *L. infantum chagasi;* **SI[g]:** *T. cruzi*; Reference drugs: **PTM**- Pentamidine, **BZND**- Benznidazole, **ARTN**- Artemisinin, **NT**- Not tested, **NC**- Not calculated.

Chemical compounds isolated from plant extracts were studied because of their proven *in vitro* antiprotozoal activity, including the molecules derived from CinAc[11]. Among the synthetic analogs of CinAc, E5 presented the lowest IC_{50} value against *L. infantum chagasi* (212.75 μM); when compared to *L. braziliensis* (IC_{50} 340.43 μM). When tested against *T. cruzi*, IC_{50} was 1071.5 μM. Another analog, I9, presented IC_{50} value of 248.56 μM against *L. braziliensis*; against *L. infantum chagasi* and *T. cruzi*, I9 showed IC_{50} values ranging between 582 and 723 μM. Analog G7 showed inhibitory concentration against *L. infantum chagasi* (IC_{50} 642.9 μM) and against *T. cruzi* (IC_{50} 667.11 μM). Analogs A1, B2, C3, D4, and F6 were not effective at a concentration of 1,260 μM **(Table 1)**. The analogs, at concentration of 1,260 μM (the highest concentration tested), presented no antimalarial, hemolytic, or cytotoxic activity; thus, selectivity index calculations were based on this concentration.

Analogs E5 (*L. infantum chagasi*) and I9 (*L. braziliensis*) presented the best IC_{50} of 212.7 and 248.5 μM, respectively. When we analyzed the leishmanicidal action among parasite species in relation to the treatment control pentamidine, greater efficacy was obtained against *L. braziliensis* (IC_{50} 0.76 μM) than against *L. infantum chagasi* (IC_{50} 1.4 μM).

Studies have described the fractionation of biomonitoring of *Valeriana wallichii* chloroform extract, which resulted in two active CinAc derivatives against *L. major* promastigotes with IC_{50} of 48.8 μM[11]. In this context, 3-(3,4,5-trimethoxyphenyl) propanoic acid obtained naturally from *Piper tuberculatum* Jacq, also presented activity against *L. amazonensis* promastigotes after 96 h hours of treatment[6] with IC_{50} at the concentration of 145 μg/mL (608.63 μM). In another study, synthetic derivatives of CinAc (3,4,5-trimethoxicinnamic acid) were effective at the concentration of 2 mg/mL (8.39 mM) against promastigotes of *L. amazonensis*, causing death of 92% of the parasites when treated for 96 h[12]. The results of this study corroborate the results presented here, where the tests against *Leishmania* promastigotes were performed for 72 h and the maximum concentration was 1,260 μM.

The CinAc derivatives were inactive against the W2 (CQ-resistant) *P. falciparum* strain (IC_{50} > 1,260 μM). However, we expected high activity for this class since CinAc isolated from *K. africana* (β-hydroxycinnamic) showed IC_{50} ranging from 53.84 to 6.71 μM against other *P. falciparum* strains (W2, W2mef, CAM10, and SHF4)[13]. The main mechanism of antiplasmodial action of CinAc class is the decline in energy production (ATP) due to inhibition of lactate transporters, changes in mitochondrial respiration, and reduction of translocation of carbohydrates and amino acids in parasitized red blood cells. Previous studies have frequently suggested that structural modifications of CinAc may alter its effectiveness, thus, reducing its antiplasmodial activity[14].

Derivatives of natural products of plant origin exhibiting trypanocidal activity have been considered valid alternatives for control of parasites[15]. In this context, studies performed with the species *P. arboreum* and *P. tuberculatum*, using the hexane extracts obtained from leaves against *T. cruzi* epimastigotes, resulted in an IC_{50} of 13.3 μg/mL for *P. arboreum* and 17.2 μg/mL for *P. tuberculatum*[5]. Among the CinAc analogs tested, G7 showed the best trypanocidal activity, exhibiting IC_{50} of 667.1 μM and selectivity index of 1.9.

The results demonstrated that synthetic analogs of CinAc have antiprotozoal activity against *L. braziliensis* and *L. infantum chagasi* promastigotes, and *T. cruzi* epimastigotes. However, prior to performing the *in vitro* and *in vivo* tests, it is suggested that the compounds undergo structural modifications in order to obtain a more satisfactory selectivity index. The analogs tested are promising in the development of new molecules with greater effectiveness and fewer side effects.

Acknowledgement

The authors express their gratitude to Amy Grabner for the English review of the present manuscript, Malaria and Leishmaniasis Bioassay Platform (PBML) - FIOCRUZ-RO and Federal University of Rondônia for the opportunity of carrying out the research.

Conflict of interest

The authors declare that there is no conflict of interest.

Financial Support

This work was supported by Coordination of Improvement of Higher Level Personnel (CAPES) and Institute of Epidemiology of Western Amazonia (INCT-EpiAMO).

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