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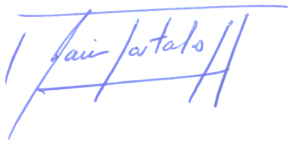
Yo, Dr. Jaime Costales Cordero, certifico que la disertación de Microbiología del candidato Eloy Dylon Ramos Dussling ha sido concluida de conformidad con las normas establecidas; por lo tanto, puede ser presentada para la calificación correspondiente.



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DIRECTOR DE LA DISERTACIÓN

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Para mis padres por siempre estar

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Evaluation of the in vitro anti-*T.cruzi* activity of four antimicrobial peptides from Ecuadorian anurans

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Abstract

Background: Chagas disease (CD) caused by *Trypanosoma cruzi*, is considered the most important parasitic disease in the Americas. It is estimated to affect approximately 8 million people, 30% of which will develop the debilitating and potentially fatal health complications as a result. Infection by *T. cruzi* can be cured if treatment with benznidazole or nifurtimox is administered shortly after the infection occurs. However, treatment efficacy decreases over time, making treatment of chronic Chagas disease challenging. Additionally, these drugs frequently induce side effects. Antimicrobial peptides (AMPs), biologically active molecules produced by organisms through their innate immune system, are of considerable interest for developing new antimicrobial therapies.

Methods. The anti-*T. cruzi* activity of four antimicrobial peptides derived from the gliding tree frog, *Agalychnis spurrelli*, was studied. Synthetic versions of adenoregulin-AS1 (ADN-AS1), dermatoxin-AS1 (DTX-AS1), dermaseptin-SP9 (DRS-SP9), and dermaseptin-SP10 (DRS-SP10) were tested *in vitro* against trypomastigotes of the recombinant Tula-ß-gal *T. cruzi* strain via colorimetric assays. The cytotoxicity of the peptides against mammalian cells was also evaluated, using rezasurin reduction assays.

Results: The four studied peptides exhibited varying degrees of activity against *T. cruzi* tripomastigotes, the parasite forms infective to mammals, including humans. Three peptides (DTX-AS1, DRS-SP10, and ADN-AS1) displayed activity against the parasite (EC50 = 0,35, 1,21 and, 1,58 µM, respectively) with limited cytotoxic effects over mammalian host cells. DRS-SP9, conversely, displayed much lower (EC50 = 22, 91µM) activity.

Conclusions: These findings suggest the studied peptides are active against *T. cruzi* trypomastigotes. Dermatoxina-AS1 (DTX-AS1) displays the most specific activity

against the parasite. Although our data suggest peptides are lytic for the parasite, additional studies are required to clarify their mechanism of action.

Key words: Chagas disease, peptides, cytotoxicity, *Trypanosoma cruzi*.

Background

Chagas disease (CD) is a zoonotic disease caused by the protozoan parasite *Trypanosoma cruzi* and is considered the most important among the parasitic diseases in the Americas [1]. It is estimated that CD affects around 8 million people, 30% of whom will develop will develop debilitating and potentially fatal health complications because of the infection with the parasite [2].

T. cruzi infection is transmitted through the activity of hematophagous insect vectors, known as triatomine bugs or "kissing bugs" [3]. Additionally, it can be transmitted congenitally, through contaminated organ transplants, transfusion of blood contaminated units, orally, and through laboratory accidents [4].

CD presents two phases known as acute and chronic. The acute phase begins at the time of infection and lasts approximately two to three months [5]. Generally, this phase is asymptomatic or exhibits nonspecific symptoms, such as elevated body temperature, aching head, swollen lymph nodes, paleness, discomfort in the muscles, breathing difficulties, inflammation, and experiencing pain in the abdomen or chest, as well as inflammation at the site of parasite entry known as Romaña's sign [5], [6].

The chronic phase of the disease starts two to three months after the initial infection when parasitaemia levels become undetectable by microscopy, and the remaining parasites remain hidden in tissues and organs [6], [7]. Clinical manifestations of the chronic phase may appear after several years, and it is estimated that 30% of infected individuals will develop Chagas cardiomyopathy. Characteristic conditions of Chagas cardiomyopathy include arrhythmias, heart block, and apical aneurysm. Patients may experience palpitations, pulmonary embolism, and have a high risk of sudden death or death due to advanced hear failure [8].

The life cycle of the parasite begins when a triatomine bug feeds on blood contaminated with *T. cruzi* and establishes itself as epimastigotes in the bug's midgut. They undergo longitudinal binary fission and adhere to peri microvillar membranes secreted by epithelial cells. Once in the rectum, some of the epimastigotes differentiate into metacyclic trypomastigotes, which are infectious to mammals and are eliminated in the bug's faces and urine [9].

From there, the parasites enter the bloodstream through the bite wound and are transported to tissues and organs where they infect nucleated cells [4]. Inside the cells, they differentiate into amastigotes to replicate. Once the cell is filled with parasites, the amastigotes differentiate back into trypomastigotes, which break the cell membrane and continue infecting new cells [7] or are taken up from the bloodstream by a new vector.

Infection caused by *T. cruzi* can be cured if treatment with benznidazole or nifurtimox is administered shortly after the infection occurs. However, the efficacy decreases over time [1]. It is worth noting that these medications cause a significant number of side effects such as anorexia, weight loss, polyneuropathy, nausea, vomiting, headaches, dizziness, and they have low effectiveness in the chronic phase [10]. For this reason, the research of new extracts from different plants is crucial for the development of effective drugs to counteract CD.

For the present study, four peptides derived from anuran cutaneous skin secretions to study their activity against *T. cruzi*. The peptides under investigation were Adenoregulin-AS1 (ADN-AS1), dermatoxin-AS1 (DTX-AS1), dermaseptin-SP9 (DRS-SP9), and dermaseptin-SP10 (DRS-SP10). Antimicrobial peptides (AMPs) are biologically active small molecules produced by various organisms as part of their innate immune system. They have become a topic of considerable interest in the search for new therapies [11]–[13].

The mechanism of action of antimicrobial peptides is mainly direct lysis. While their antibacterial activity has been primarily studied, it has also been demonstrated that they exhibit diverse activities against microorganisms, such as antifungal, antiviral, and antiparasitic effects, as well as actions against tumour cells, presenting antitumor properties [13], [14]. The significance of these peptides lies in their alpha-helical amphipathic structure, which allows them to interact with membrane bilayers. Therefore, it is essential to investigate the different peptides to demonstrate their importance in addressing microorganisms that affect humans [11].

A notable example is dermaseptin, which has shown activity against multidrug resistant bacteria [15]. Furthermore, it has been demonstrated to be effective against *Candida albicans* by inhibiting its growth and biofilm formation [16]. This peptide is also being tested as an inhibitor of the initial phase of COVID-19 infection, as it interacts with spike proteins to block their binding to ACE2, potentially having therapeutic applications [17]. Lastly, dermaseptin has been extensively investigated in relation to cancer cells [18].

In the case of dermatoxin, it has been found to have antibacterial potential, particularly against gram-positive bacteria, due to its ability to disrupt the plasma membrane [19], [20]. On the other hand, adenoregulin has demonstrated lethal effects against filamentous fungi and a broad spectrum of pathogenic microorganisms. Additionally, recombinant strains of *Escherichia coli* are being generated for large-scale production of this peptide for subsequent use as an antimicrobial agent [21], [22].

Here, we have evaluated the trypanocidal and cytotoxic activity of different peptides derived from the Ecuadorian anuran *Agalychnis spurrelli*. Their half-maximal effective concentration (EC50) against trypomastigotes and half cytotoxic concentration (CC50) against mammalian cells was assessed to determine their specific anti-*T. cruzi* activity.

Methods

Cell culture

LLCMK2 monkey kidney cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (DMEM 10). The cells were maintained at 37°C with 98% relative humidity and 5% carbon dioxide (CO_2) [23].

Parasite culture

T. cruzi trypomastigotes from the recombinant Tula-ß-gal strain expressing ß-galactosidase were produced by infecting the LLC-MK2 cell line in cell culture flasks. The cells were infected with 1x10^6 trypomastigotes in 10 ml of DMEM supplemented with 2% FBS and 1% penicillin/streptomycin (DMEM 2) for 48 hours. The incubation was carried out at 37°C with 98% relative humidity and 5% CO_2. After 5 days of infection, trypomastigotes were collected and counted microscopically using a hemocytometer.

Peptides

We utilized synthetic versions of peptides, namely Adenoregulin-AS1 (ADN-AS1), dermatoxin-AS1 (DTX-AS1), dermaseptin-SP9 (DRS-SP9), and dermaseptin-SP10 (DRS-SP10), which were manufactured by BIOMATIK, Ontario, Canada. These peptides exhibited an impressive purity level surpassing 94% (23).

The Tocris Molarity Calculator (https://www.tocris.com/resources/molarity-calculator) was employed to perform peptide dilution calculations, according to peptide molecular weight, and the corresponding dimethyl sulfoxide (DMSO) volume required. Finally, 10 µL of the prepared solution were transferred to 0,5 ml Eppendorf tubes and

142 frozen at 20 ºC until use.

143 **Cytotoxicity assay**

144 Cell viability was measured by resazurin (RZN) reduction fluorescence assay, aiming to
145 evaluate whether the different peptides exhibit cytotoxic activity against LLCMK2
146 monkey kidney cells of *Macaca mulatta*. In a 96- well black plate (Thermo Scientific),
147 2x10^4 cells per well were cultured and allowed to attach overnight. Subsequently, three
148 washes with PBS were performed and 100 µL of DMEM without phenol red or FBS
149 (DMEM – PR) were added to each well.

150 As a first step, peptides were evaluated at concentrations ranging from 100 µM to
151 0,19 µM on LLC-MK2 monkey kidney cells. Dilutions were prepared in DMEM-PR in
152 a 200 µL volume and placed in duplicate wells. Control wells without peptide were
153 included. Subsequently, 10 µL of 3 mM resazurin sodium salt (RZN) in PBS were added
154 to each well and the plate was incubated for 24 hours at 37ºC with 5% CO_2 and 98%
155 relative humidity. Fluorescence readings of the plate were taken at 24 hours using a
156 GloMax multimodal microplate reader (Promega) with excitation at 530-560 nm and
157 emission at 590 nm.

158 **Evaluation of peptide anti-*T. cruzi* activity**

159 To determine if the studied peptides have activity against *T.cruzi*, colorimetric assays [24]
160 were performed. Parasites were collected, pelleted by centrifugation, and incubated for
161 two hours to allow trypomastigotes to emerge from the pellet into the culture medium.
162 Trypomastigotes were counted in a Neubauer chamber. One million parasites were placed
163 per well in DMEM-PR and exposed to peptide serial dilutions (100 µM- µ0,09 µM range),
164 also prepared in DMEM-PR. Subsequently, 25 µL of 500 mM Chlorophenol red-β-D-
165 galactopyranoside (CPRG) were added to each well. Four hours later, absorbance was

measured 600 nm with a GloMax multimodal microplate reader (Promega). Each assay was performed in duplicate. Three independent replicates of each assay were performed.

Statistical Analysis

Data analysis was performed using the half-maximal effective concentration (EC50) and half-maximal cytotoxic concentration with GraphPad Prism software (Version 8.0.1), employing the log(inhibitor) vs. response -- Variable slope (four parameters) model.

Results Peptide cytotoxicity

Results from the cytotoxicity analysis are displayed in Figure 1a and Table 1. Peptides demonstrated variable degrees of cytotoxicity over LLC-MK2 monkey kidney cells. DRS-SP9 displayed low cytotoxicity since cell viability remained close to 100% at all concentrations. Despite a CC50 being mathematically calculable, at 29,91 μM, it is obvious from the viability curves this peptide is not cytotoxic against LL-MK2 cells. ADN-AS1 also displayed low cytotoxicity, with a CC50 of 72,30 μM. DTX-AS1 and DRS-SP10 displayed a moderate cytotoxic effect (31,26 and 20,81 μM, respectively) (Table 1).

Table 1. Effect of studied peptides over mammalian cells and *T. cruzi* trypomastigotes.

Peptide	CC50 (± SD)	EC50 (± SD)	Selectivity index
ADN-AS1	72,30 (± 1,00)	1,58 (±0,01)	45,75
DTX-AS1	31,26 (±1,11)	0,35 (±0,02)	89,31
DRS-SP9	29,91 (±1,25)	22,68 (±0,04)	1,31
DRS-SP10	20,81 (±1,57)	1,21 (±0,01)	17,19

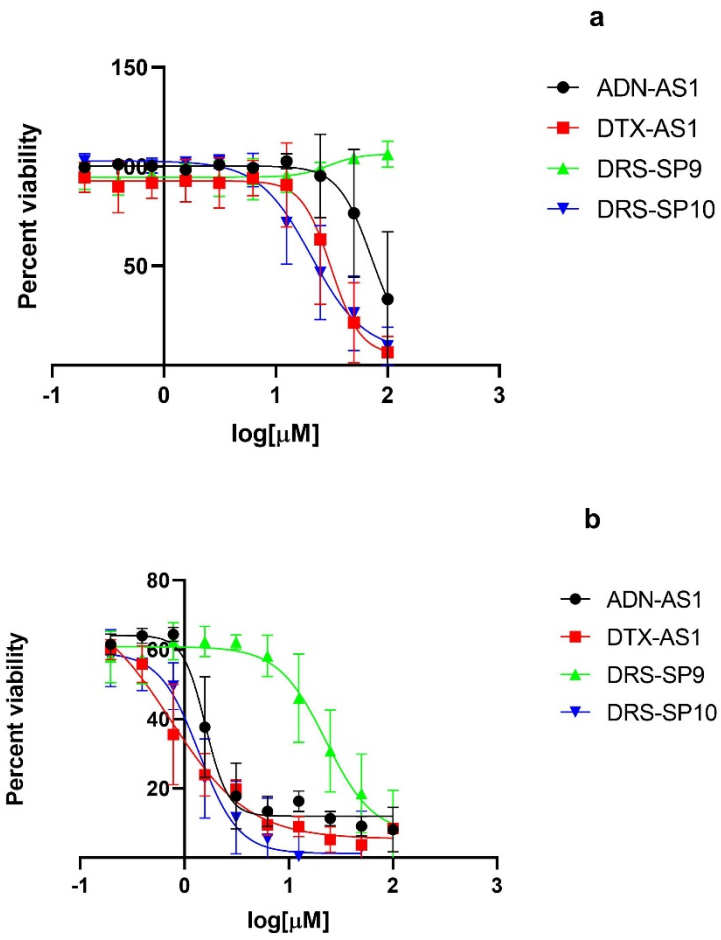

Fig 1. Effect of adenoregulina-AS1 (ADN-AS1), dermatoxina-AS1 (DTX-AS1), dermaseptina-SP9 (DRS-SP9), and dermaseptina-SP10 (DRS-SP10) over mammalian cells and *T.cruzi* tripomastigotes. Peptides were tested at concentrations ranging from 100 μM to 0,009 μM. **a**. LLC-MK2 monkey kidney cells were exposed to peptides for 24 hours; viability was evaluated via RZN reduction (Methods). **b**. *T. cruzi* trypomastigotes were exposed to peptides for four hours, parasite viability was assessed via a CPRG-based colorimetric assay (Methods).

Peptide activity over *T.cruzi* trypomastigotes

Results are displayed in Figure 1b and Table 1. DTX-AS1 displayed the most activity

against *T. cruzi* (EC50= 0,35μM). Remarkably, the activity of this peptide over mammalian cells (CC50=31,26 μM) was 89 times less intense, indicating it is highly selective towards *T. cruzi*. DRS-SP10 and ADN-AS1 also displayed strong anti-*T. cruzi* activity (EC50= 1,58 μM and 1,21, respectively), with relatively lower specificity, as suggested by their selectivity indexes (17,19 and 45,75, respectively). Finally, DRS-SP9 displayed low anti-*T. cruzi* activity.

Discussion

Treatment options for Chagas disease are scarce and not optimal. The currently approved medications, nifurtimox and benznidazole, do not always produce a complete cure and yield several undesirable side effects [25]. Parasitological treatment during the chronic phase of the disease poses additional challenges, and chronic damages to organs cannot be reverted by eliminating the parasite Therefore, identifying molecules which possess anti-*T. cruzi* activity is paramount.

In this context, the study of antimicrobial peptides and their activity over *T. cruzi* could provide insights into new treatment options. Identifying peptides that display specific lytic activity against *T. cruzi* without causing significant damage to mammalian cells would indicate such peptide might be developed into a therapeutic alternative. The peptides primarily act on cell membranes or specific components [26]. In the case of Chagas disease, which involves a systemic infection and intracellular parasite forms (amastigotes), intracellular peptide delivery is an important challenge to be overcome, which would limit use of the peptides as treatment.

Here, we have studied the activity of four peptides derived from skin secretions form the gliding tree frog, *Agalychnis spurrelli*, namely Adenoregulina-AS1 (ADN-AS1), dermatoxina-AS1 (DTX-AS1), dermaseptina-SP9 (DRS-SP9), dermaseptina-SP10

(DRS-SP10, against *T cruzi* trypomastigotes. Trypomastigotes are the parasite life cycle stage capable of infecting mammals, including human beings. Many previous studies reporting the activity of anti-*T. cruzi* agents have been performed on epimastigotes [27], [28]. Although epimastigotes are easier and more cost-effective to culture, they represent the life cycle stage present in the vector, non-infectious to humans. Therefore, we chose to perform the study using trypomastigotes, which are more relevant to human infection. To evaluate whether the lytic activity is specific to *T. cruzi*, the cytotoxicity over LLC-MK2 cells was also measured.

Three studied peptides displayed different degrees of cytotoxic activity against LL-MK2 cells: moderate toxicity for DTX-AS1 and DRS-SP10, and low citotoxity for ADN-AS1. Conversely, DRS-SP9 did not lyse the cells at the concentrations tested. The ability of these peptides to discriminate between mammalian cells and microbial cells have been known for over ten years [29], [30]. This is thanks to the amino acid composition of the peptides and additionally, to the physicochemical properties of the membranes [29], [30], which is consistent with the results obtained with the different studied peptides.

Previous studies have demonstrated that anuran peptides have the ability to selectively interact with the membranes of microbial cells, such as bacteria and parasites, while showing lower affinity for mammalian cell membranes [31]. This selectivity is attributed to the unique amino acid composition of the peptides and their physicochemical properties, such as electric charge and hydrophobicity [31]. These characteristics allow them to specifically bind to the membranes of microorganisms, disrupt their integrity, and compromise vital functions, leading to their death. On the other hand, mammalian cells have membranes with different compositions and distinct properties, limiting the interaction of the peptides and reducing their toxicity towards these cells.

These findings support the idea that anuran peptides could be used as selective therapeutic agents to combat microbial infections without causing significant harm to host cells. Dathe, et al., [32] investigated the relationships between the membrane-binding properties and cytotoxicity of cationic lytic peptides. Their study revealed that cytolytic activity correlates primarily with membrane insertion affinity, which subsequently provides protection against enzymatic cleavage of red blood cells. These findings suggest that the cytotoxicity of anuran skin peptides might involve their interaction with cell membranes and disruption of vital cellular processes, leading to cell death [32].

Anuran peptides are produced in the granular glands of amphibians' skin and play an important role in their innate immune response. These peptides provide protection against pathogenic microorganisms [33]. Due to this capability, peptides represent a potential alternative for future treatments against these microorganisms.

In the case of the four anuran peptides used in our study (ADN-AS1, DTX-AS1, DRS-SP9, DRS-SP10), intense activity against *T. cruzi* trypomastigotes was observed for all peptides except DRS-SP9. This is consistent with various studies in which peptides, such as antimicrobial peptides (AMPs), have been evaluated for anti-trypanosomal activity. In these studies, the peptides were tested at different concentrations and showed activity against both trypomastigotes and epimastigotes [34]– [37].

The validity of using anuran peptides against *T. cruzi* is further supported by reports indicating antimicrobial peptides isolated from *Phyllomedusa nordestina* (Amphibia) displayed an IC50 value of 10 μM against trypomastigotes [38]. Therefore, the peptides analysed in our study could potentially be more efficient in inhibiting *T. cruzi*, as we have lower values, such as DTX-AS1with an EC50 value of 0,35 μM.

Our data suggests the activity of peptides (ADN-AS1 and, especially, DTX-AS1

267 is highly selective for *T. cruzi*. This is supported by previous reports indicating low
268 toxicity for peptides derived from amphibians against mammalian cells [39].

269 A detailed *in silico* analysis of the chemistry of the studied peptides and their
270 interaction with the parasite plasmatic membrane (predicted secondary structures,
271 docking scores, among other parameters) might provide additional insights into the
272 mechanism of action. β- galactosidase is an intracellular enzyme [39], and its activity
273 over CPRG in the culture medium indicates that the mechanism of action of the studied
274 peptides involves trypomastigote lysis. It remains to be determined whether the studied
275 peptides are active against intracellular amastigotes, responsible of parasite replication
276 during human infection. Any candidate for Chagas disease treatment must be able to act
277 over trypomastigotes as well as intracellular forms of the parasite. The latter is more
278 challenging because it involves the capacity to cross the plasma membrane of host cells.
279 Additional studies are required to clarify whether the studied peptides have this capacity.
280 Anuran skin secretion remains a valuable source of bioactive compounds, which can be
281 surveyed for their activity against pathogens.

282 ## Conclusion

283 We have determined three out of four novel peptides derived from Ecuadorian anurans
284 are selectively toxic against *T. cruzi* trypomastigotes *in vitro*. They exhibit EC50 values
285 comparable to those reported in the literature and show a moderate to low level of
286 cytotoxicity against monkey kidney LLCMK2 cells. Further testing and development of
287 these compounds are justified, including testing over intracellular amastigotes and testing
288 on different *T. cruzi* strains.

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