Pontificia Universidad Católica del Ecuador

Facultad de Ciencias Exactas y Naturales

Carrera de Microbiología

Trypanocidal activity of dermaseptins isolated from *Agalychnis spurrelli* (Anura: Hylidae) skin secretion

Disertación previa a la obtención del título de Microbiólogo

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Quito, 2021

Yo, Mtr. Miryan Rivera, certifico que la disertación de Microbiología del candidato Mateo Alejandro Salazar Salazar ha sido concluida de conformidad con las normas establecidas; por lo tanto, puede ser presentada para la calificación correspondiente.

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DIRECTORA DE LA DISERTACIÓN

Quito,

Para mi hermana Daniela, por no dejarse vencer

Agradecimientos

Agradezco al Centro de Investigación para la Salud en América Latina (CISeAL), por permitirme realizar mi trabajo de disertación en sus instalaciones y a la Pontificia Universidad Católica del Ecuador por el financiamiento mediante el proyecto QINV0209-IINV529010100.

A la Mtr. Miryan Rivera Íñiguez, por su apoyo, su confianza y su guía a lo largo de mi formación académica y por incluirme en su grupo de investigación del Laboratorio de Investigación en Citogenética y Biomoléculas de Anfibios (LICBA). Igualmente, al Dr. Jaime Costales Cordero por su guía y apoyo en mi trabajo de disertación ya que, sin su ayuda, esta investigación no hubiera sido posible. A la Dra. Marbel Torres por su ayuda y apoyo en este proyecto con las fotografías de microscopía electrónica, ya que sin ella esto no sería posible.

A Ailín Blasco por su apoyo incondicional en cada momento de mi desarrollo académico y su guía para que pueda realizarse este trabajo. A Carolina Proaño, quien, con sus investigaciones previas sobre dermaseptinas, hizo posible la ejecución de esta disertación.

Agradezco a mis profesores de Microbiología, en especial a la Mtr. Magaly Estrella, a Mtr. Jenny Yánez y a la Mtr. Elena Granda por ser siempre las que me ayudaron y apoyaron ya que sin ellas no podría ser la persona que soy ahora.

A mi mamá, mi papá, mi hermana, mi tía y mis abuelitos por su apoyo incondicional en mi desarrollo a nivel de persona y académico. A mis amigos de toda la vida, en especial a David y Gabriela por estar siempre en mis momentos más difíciles. A mis amigos de carrera y que hice a lo largo del transcurso de mis estudios por brindarme su ayuda, agregar nuevas experiencias a mi vida y ser mi apoyo, especialmente a Andrea, Michelle, Patricia, Sebastián, Renata, Salomé, Johanna, Melanie, Esteban, Jess, Javier y Josué por ayudarme en el desarrollo de este proyecto.

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TITLE

Trypanocidal activity of dermaseptins isolated from skin secretion of *Agalychnis spurrelli* (Anura: Hylidae)

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Abstract

Chagas disease (CD) is a serious health problem worldwide, affecting between 6 and 7 million people, especially in Latin America. This tropical disease, caused by the protozoan parasite *Trypanosoma cruzi*, can be asymptomatic during the acute phase (first two months of infection), while in the chronic phase irreversible damages to the heart and/or digestive system develop in 30-40% of those infected. The drugs currently used to treat CD are not always effective and they cause side effects that lead patients to abandon treatment; therefore, new therapeutic options are required. In this work, we evaluated the antiparasitic and cytotoxic activity of three antimicrobial peptides of the dermaseptins (DRS) family (DRS-SP2, DRS-SP4 and DRS-SP5) obtained from the cutaneous secretion of the frog *Agalychnis spurrelli*, against the trypomastigote and amastigote stages of *T. cruzi*, Tulahuen-β-gal strain, which express β-galactosidase. In conclusion, β-

galactosidase activity measurements, oxidation-reduction of resazurin and electronic microscopy analysis confirmed that DRS-SP2, DRS-SP4 and DRS-SP5 are capable of lysing both, extracellular (IC_{50}s 1.26, 2.31 and 19.0, respectively) and intracellular parasites (IC_{50}s 5.85, 28.07 and 14.59, respectively), while the DRS-SP4 has the highest ratio (14.14) between parasite/host cell cytotoxicity.

Introduction

Trypanosoma cruzi is the causative agent of Chagas disease (CD). This parasite is widely distributed in Latin America (LA) and CD cases have also been reported in the United States, Canada, many European countries, and the Western Pacific (1). Human migration from impoverished regions in LA to developed countries is believed to be a major factor driving the globalization of this disease (2). In non-endemic regions, blood transfusions and congenital transmission increase cases in non-endemic regions are important means of transmission (2,3).

CD occurs in two distinct phases: acute and chronic. In the acute phase, which takes place during the first two months post- infection, the available etiological treatment is more likely to be successful. In the chronic phase, around 30-40% of infected individuals develop irreversible heart and/or digestive health damages, often resulting in severe disability and death (4).

CD is usually treated with benznidazole (BZN), nifurtimox and fexinidazole, none of which is completely effective. During the acute phase, BZN is 70-80% effective if it is provided to children under 12 years of age, in laboratory accidents, and in congenital infection cases (1,5–7). In newborns, BZN is 90% effective during the first year of life (4,5).

In the chronic phase, children are treated in similar fashion as in the acute phase (5,8–10). However, adults have lower likelihood of recovery (~10%), and antiparasitic drugs are used to

prevent or reduce the development of more serious forms the disease, such as cardiomyopathy, and to avoid congenital transmission. Otherwise, both available drugs induce adverse effects, such as anorexia, weight loss, nausea, vomiting, peripheral neuropathy, leukopenia, insomnia, spasms, paresthesia, polyneuritis, and convulsive seizures (5,8).

Because of afore mentioned limitations in terms of efficacy and side effects of the currently available drugs employed to treat CD, it is necessary to develop new treatments that can be used in any of the two phases of the disease and that do not cause collateral damage to the patient (5). In this context, the study of antimicrobial peptides with demonstrated *in vitro* activity against *T. cruzi* could potentially provide insights into novel treatment strategies for CD (11). For example, AMPs Temporizin and Temporizin-1, that were obtained from *Rana temporaria*, were found to be highly toxic against *T. cruzi*, inducing chromatin condensation, mitochondrial cristae disorder, kinetoplast disorganization and reservosome swelling, without significant cytotoxicity against macrophages J774 (12). Additionally, phylloseptins, peptides obtained from the frog species *Phyllomedusa nordestina* (13), yielded IC_{50} of 0.25–0.68 μM values against *T. cruzi* without significant cytotoxicity against mammalian cells (13).

In this study we have analyzed the effects of three different dermaseptins (DRS-SP2, DRS-SP4, DRS-SP5) against *T. cruzi*, mammalian cells, mammalian cells infected with the parasite. Dermaseptins are amphipathic polycationic, α-helical molecules, with 28-34 residues in length and have positive net charge. This family of antimicrobial peptides that are present in the skin secretions of several tree frogs from the Hylidae family, especially from *Agalychnis* and *Phyllomedusa* genus (24). Previously, these molecules were tested against bacteria (*Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923) and yeast (*C. albicans* clinical isolated), showing that DRS-SP2 had bioactivity against both bacteria (minimal inhibitory

concentration (MIC): 2.67 µM), and also against *C. albicans* (MIC: 10.70 µM) (25). Understanding the effects of antimicrobial peptides over *T. cruzi* and, specially, the mechanisms by which they alter the parasite´s membrane, may open avenues to search for novel approaches for parasitological treatment of CD.

Material and Methods

Reagents

All reagents were purchased from GIBCO (Grand Island, NY, U.S.A) and SIGMA (St. Louis, MO, U.S.A).

Cell culture

Monkey kidney cells ($LLcMK_2$) from *Macaca mulatta,* were maintained in Dulbecco Modified Eagle's Medium (DMEM) supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum (FBS) (DMEM 10), at 37 °C with 5% CO_2 atmosphere and 98% relative humidity. Subcultures were performed weekly.

Parasite culture

T. cruzi strain Tulahuen (clone C4; +lacZ, hence forth abbreviated Tula β-gal) trypomastigotes were used to infect $LLcMK_2$ cell cultures. Monolayers were infected with $5x10^5$ trypomastigotes in 10 ml of DMEM supplemented with 2% FBS, 1% penicillin/streptomycin (DMEM 2) and incubated for 48 hours at 37 °C with 5% CO_2 atmosphere and 98% relative humidity. Subsequently, the cell monolayer was washed with phosphate-buffered saline (PBS) to eliminate parasites and 10 ml of DMEM 2 were added. Five days after infection, trypomastigotes were harvested from the culture to use in the different assays.

Antimicrobial peptides dermaseptin

Synthetic versions of DRS-SP2, DRS-SP4 and DRS-SP5 were produced by BIOMATIK (Ontario, Canada) with a purity greater than 94% (23). These biomolecules were previously characterized and sequenced from the total secretion of the *Agalychnis spurrelli* tree frog and their antimicrobial activity has been previously described in the literature (23). .

Table 1. Studied peptides described in Proaño et al. (23)

Synthetic peptide	Sequence	Molecular weight	Purity (%)	Species
Dermaseptin-SP2 (DRS-SP2)	ASWKVFL KNIGKAAG KAVLNSVT DMVNQ-NH2	2989.56	97.37%	*Agalychnis spurrelli*
Dermaseptin-SP4 (DRS-SP4)	SLWSSIKD MAAAAGR AALNAVN GILNP-NH2	2711.16	96.52%	*Agalychnis spurrelli*
Dermaseptin-SP5 (DRS-SP5)	SLRSSIKD MAAAAGR AALNAVN GIVNP-NH2	2667.11	94.51%	*Agalychnis spurrelli*

Assays with *T. cruzi* trypomastigotes

Trypomastigotes were suspended in DMEM without phenol or FBS and $1x10^6$ parasites were dispensed per well, in 96-well plates and exposed to different peptide concentrations (Table 2). Chlorophenol-β-D-galactopyranoside red (CPRG) 500 µM concentration in PBS was filter-sterilized, and 50 µL added to each well. The plate was incubated at 37 °C with 5% CO_2 for 4 hours. The absorbance at 490 nm was read at 4 hours in a GloMax microplate reader (Promega). Tested peptide concentrations are shown in Table 2.

The Tula-β-gal strain expresses β-galactosidase. Upon parasite lysis, this enzyme catalyzes CPRG hydrolysis, converting this yellow-orange substrate into the red chromophore chlorophenol red, yielding a dark red solution (26), whose absorbance can be measured at 490 nm. Therefore, parasite lysis upon exposure to the peptides can be quantified by spectrophotometry and employed to calculate IC_{50} values for each peptide.

Table 2. Peptide concentrations used for determination of peptide IC_{50}s against trypomastigotes

Dermaseptin	Concentration (µM)
DRS-SP2	25, 20, 15, 12, 10, 7, 5, 3, 1, 0.5
DRS-SP4	8.5, 7.5, 6, 4.5, 3, 1.5, 0.5, 0.37, 0.25, 0.12
DRS-SP5	100, 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78, 0.39, 0.19

Tests against *T. cruzi* amastigotes

LLcMK2 cells ($2x10^4$ cells /well) were plated in 96-wells plates and incubated for 24 hours. Subsequently, the cells were infected with $1x10^5$ parasites for 24 hours in DMEM 2. The monolayers were washed four times with PBS and infection allowed to proceed for 96 hours.

Both plates were washed twice using PBS, and the concentrations of each peptide employed for the IC_{50} calculation were placed in each well as it is detailed on Table 3. Subsequently, they were incubated for 24 hours with DMEM without phenol red or FBS. After the incubation time, 50 µL of CPRG solution, containing Triton (0.5%) were added in each well. After 2 hours of incubation, the absorbance was measured at 490 nm in a GloMax microplate reader (Promega).

Table 3. Peptide concentrations used for determination of peptide IC_{50}s against infected cells

Dermaseptin	Concentration (µM)
DRS-SP2	20, 16, 13, 10, 7, 5, 3, 1, 0.5, 0.25
DRS-SP4	35, 30, 25, 20, 15, 10, 5, 1, 0.5, 0.25
DRS-SP5	100, 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78, 0.39, 0.19

Cytotoxicity tests on LLcMK2 cells

RZN reduction occurs due to the activity of the electron transport chain of the $LLcMK_2$ cells; therefore, it is directly correlated to the number of viable cells. The IC_{50}s for mammalian cell cytotoxicity for each peptide was determined based on RZN reduction. In a 96-well plate, $2x10^4$ cells/well were seeded in a volume of 200 µL of DMEM-10 and incubated for 24 hours. The peptides were diluted in 10 two-fold serial dilutions (100 µM to 0.0097 µM, Table 2). Subsequently, 10 µL of 3 mM resazurin sodium salt (RZN) in PBS were added per well and then were incubated. The fluorescence was measured after 24 hours, at an excitation wavelength of 530-560 and 590 nm emission wavelength in a GloMax microplate reader (Promega).

Electronic microscopy

Suspensions of $2x10^4$ $LLcMK_2$ cells in DMEM without phenol or FBS medium were placed in a 1.5 ml microtube with 20, 35 or 100 µM DRS-SP 2, 4 or 5, respectively. In addition, for trypanocidal activity assays, 3 $x10^6$ trypomastigotes in 1.5 ml DMEM without phenol or FBS medium were placed in microtubes with 10, 7.5 or 40 µM DRS-SP 2, 4 or 5, respectively. In both cases, controls were included where parasites or cells were treated only with DMEM without phenol or FBS medium. These dermaseptin concentrations were chosen based on the IC_{50}s

obtained for each peptide, to ensure parasite lysis and allow for imaging. The tubes were incubated for 2 hours at 37 °C with 5% CO_2 atmosphere and 98% relative humidity. Finally, the tubes were centrifuged at 860 RCF for 10 minutes, the supernatant was discarded, and each sample was resuspended with 1 ml of 3% glutaraldehyde. Images were acquired by Scanning Electron Microscope W filament (SEM) with Schottky filament resolution 1.2 nm at 30kV (Bruker).

Giemsa staining of infected cells

Three sterile circular coverslips per well were aseptically placed into two 6-wells plates and $5.5x10^5$ $LLcMK_2$ cells per well were plated. Twenty-four hours later, the cells were infected with $1.1x10^6$ trypomastigotes per well, and both plates were incubated for 24 hours under the same conditions. The following day, cells were washed with PBS and 8, 30 or 100 µM DRS-SP 2, 4 or 5, respectively, in DMEM without phenol or FBS medium, finally were added to two replicate wells. Control wells, cells were only exposed to medium DMEM without phenol or FBS were also include. Finally, the plates were incubated under the same conditions described previously and a coverslip was removed from each well at 24h, 48h and 72 hours and fixed with 4% paraformaldehyde, and stained with Giemsa.

Data analysis

The raw data were first processed in Excel and later analyzed on GraphPad Prism, version 9.0. The results were analyzed by non-parametric Mann-Whitney U and Kruskall-Wallis tests followed by a Duncan *post hoc* test.

Results

Trypanocidal activity

Trypomastigote lysis was quantified for serial dilutions of each peptide, plotted (Fig 1A) and IC_{50}s were calculated for each peptide (Table 4). DRS-SP2 displayed the highest lytic median activity, with significant differences when compared with DRS-SP5 ($p= 0.0219$, Fig 1B). Electron microscopy images showed the effects of the peptides over the structure of trypomastigotes treated (Fig 1C-F). Alterations in the cellular structure of the parasite were visible. Some of the trypomastigotes treated with DRS-SP4, had lost their flagella at 7.5 µM concentration. Some of the photos of electronic microscopy show that there are remnants of lysed parasites (Fig 1D-F).

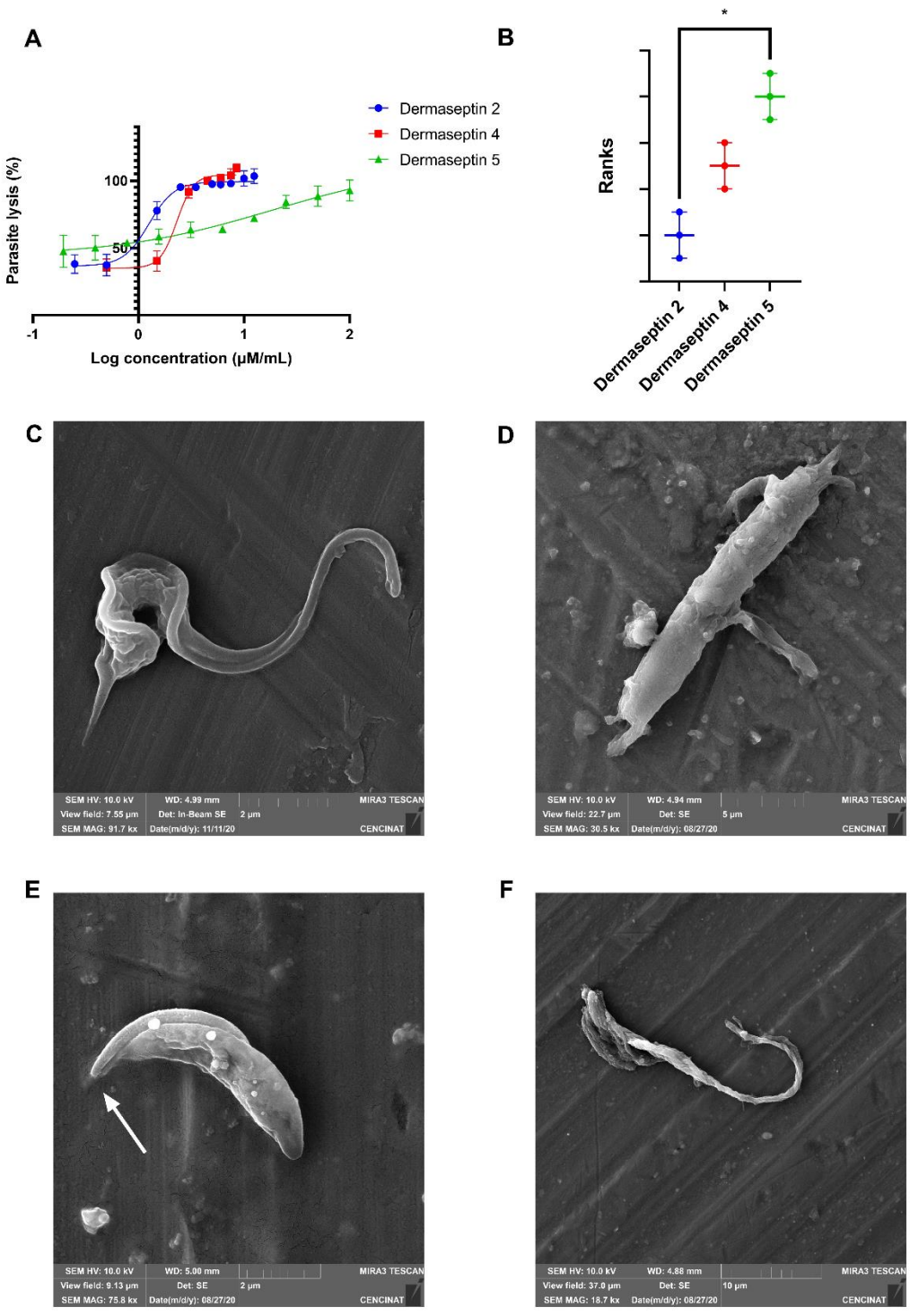

Figure 1. Trypanocidal activity of dermaseptins. **A**: Figure shows the dose-response of *T. cruzi* against the three different dermaseptins (DRS-SP2, DRS-SP4, DRS-SP5). **B**: Comparison between IC_{50} values for studied dermaseptins against *T. cruzi*. Significant differences between DRS-SP2 and DRS-SP5 found (Kruskal-Wallis test, followed by Dunn post-hoc test. p= 0.0219). **C-F** Representative electron microscopy images. **C:** Untreated parasite, showing the characteristics of

the structure of trypomastigotes. Magnification: 91,700X. **D:** Trypomastigotes treated with 10 µM DRS-SP2. Magnification: 30,500X. **E:** Trypomastigotes treated with 7,5 µM DRS-SP4 lost their flagellum (white arrow). Magnification: 75,800X. **F:** Trypomastigotes treated with 40 µM DRS-SP5. Magnification: 18,700X.

Table 4. Dermaseptin activity in each assay

DRS-SP	Extracellular effect IC_{50} (µM) [a]	Intracellular effect IC_{50} (µM) [b]	Cytotoxicity IC_{50} (µM) [c]
2	1.26	5.855	9.833
4	2.31	28.07	33.43
5	19.0	14.59	100

[a] DRS-SP5 IC_{50} is significantly different to the other two dermaseptins tested (p=0.0219, Wilcoxon statistical test).

[b] DRS-SP4 is significantly different to the other dermaseptins p=0.0219

[c] Significantly different between each other p= 0.0022

Effect of dermaseptin against intracellular parasites

The dose-response curve against intracellular parasites is shown and Fig 2A and the corresponding IC_{50}s are displayed in Table 4. Significant differences between DRS-SP2 and DRS-SP4 (p=0.0219) were detected. Fig 2C-F show Giemsa stained cells after treatment with the peptides. At the concentrations employed (Table 3), the morphology of the peptide-treated host cells is clearly affected at 24-hours post treatment. However, at 48 hours, host cells recovered from DRS-SP2 and DRS-SP5 treatment, and a reduced number of infected cells were observed on the analyzed images. On the other hand, cells treated with DRS-SP4 showed many parasites inside them at 48 hours on the photos.

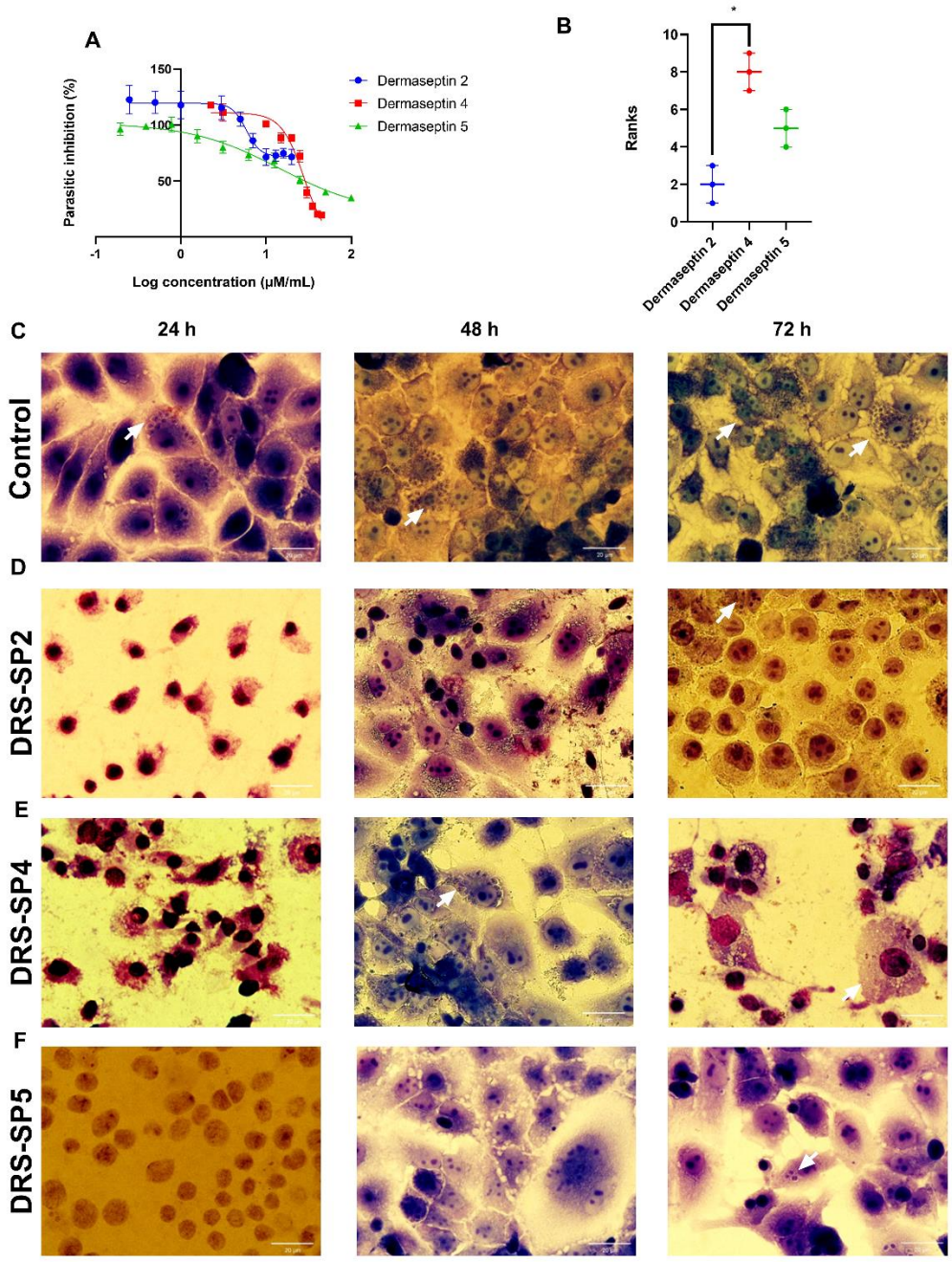

Figure 2. Effect of dermaseptins against intracellular parasites and infected cells. LLcMK$_2$ cells were infected and treated with each peptide were stained with Giemsa. Representative images are shown A: Dose-response curves with each dermaseptin are shown. **B:** Comparison between the IC_{50}s.Significant differences between DRS-SP2 and DRS-SP4 (Kruskal-Wallis test, followed by Dunn post-hoc test. p=0.0219) were found. **C-F**: Images are showing the Giemsa staining. The

magnification for all the images is 20 µm. **C**: Cells with the parasite without any treatment that correspond to the control. There are parasites inside the cells (white arrows). **D:** Infected cells treated with DRS-SP2. Red arrow marks the parasites inside the cells at 72 hours. **E:** Infected cells treated with DRS-SP4. There are parasites from 48 hours (white arrow). **F:** Infected cells treated with DRS-SP5, and there is one cell with three parasites inside (white arrow).

Cytotoxicity of DRSs over host cells

The effect of DRS-SP2, DRS-SP4 and DRS-SP5 over mammalian cells at concentrations ranging from 100 µM - 0.195 µM was evaluated. Dose response curves were generated (Fig 3) and IC_{50}s were calculated (Table 4), all peptides presented significant differences between each other ($p<0.0022$).

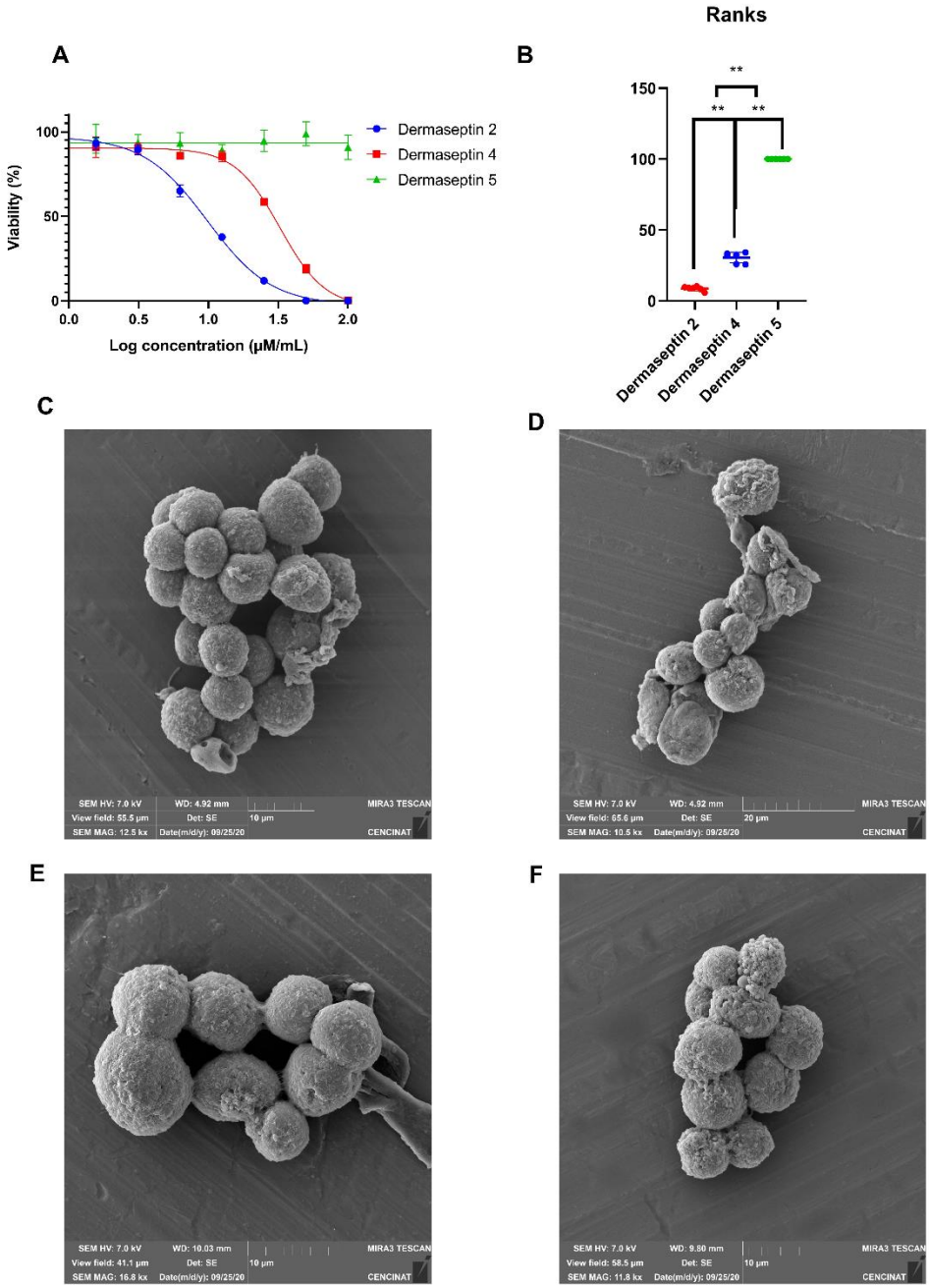

Figure 3. Cytotoxic activity of dermaseptins. LLcMK2 cells were tested against the three dermaseptins to determinate the IC_{50}. Then, the cells were treated with the IC_{50} concentration of each peptide, and then were fixed with 3% glutaraldehyde to see the effect of each peptide with electron microscopy. The experiment was independently replicated three times. **A:** Graphic represents the dose-response of LLcMK2 against the three different dermaseptins (DMS-SP2, DMS-SP4, DMS-SP5). **B:** IC_{50} value comparison for dermaseptins. A Mann-Whitney U-test was employed to compare DMS-2 vs. DMS-4 p = 0.0022, DMS-SP4 vs. DMS-SP5 p = 0.0022; and DMS-SP2 vs DMS-SP5 p = 0.0022. **C:** Untreated cells, showing the characteristics of the structure of normal cells in DMEM clear. Magnification: 12,500X. **D:** Cells treated with DMS-SP2 in 20

µM concentration and, it not presented changes. Magnification: 10,500X. **E:** Cells treated with DMS-SP4 in 35 µM concentration, and it did not present changes. Magnification: 16,800X. **F:** Cells treated with DMS-SP5 in 100 µM concentration, and it did not present changes. SEM magnification: 11,800X.

Discussion

Anuran skin secretions contain a complex assortment of biomolecules, including proteins, peptides, steroids, alkaloids, and opioids (14), some of which hold potential biomedical uses. For example, antimicrobial peptides (AMPs) are biomolecules with a variable number of monomers comprising from 9 to 100 aminoacids (15). AMPs are present in many organisms, including bacteria and more complex organisms, such as vertebrates (16). Historically, gramicidin was the first AMP discovered that was isolated from the bacteria *Bacillus sp.*, and *in vivo* experiments using this molecule demonstrated that mice were protected against pneumococcal infections (17).

Another example is pexiganan (MSI 78), which is the first synthetic cationic AMPs extracted from the African frog species *Xenopus laevis*, and it possesses *in vitro* activity against gram-positive and gram-negative bacteria (18–20). Biogels containing pexiganan in conjunction with the bacterial antimicrobial peptide nisin, that is a polycyclic peptide antibiotic, and it has been used as complementary treatment for polymicrobial diabetic foot infections (21).

Drugs currently available for Chagas disease parasitological treatment are limited and highly toxic, producing strong side effects such as nausea, dizziness, vomiting, etc., which frequently induce patients to discontinue treatment (5,27). In addition, there is natural resistance by the parasite to drugs such as benznidazole (BZN) and nifurtimox, resulting in treatment failure (28,29). Therefore, it is imperative to identify molecules with antiparasitic potential, which could

be developed into alternative treatments to control Chagas disease (30,31). We have explored the effects of novel antimicrobial peptides, specifically dermaseptins, over *T. cruzi*.

The three dermaseptins analyzed are highly efficient lysing *T. cruzi* trypomastigotes. Among them, the DRS-SP2 stands out, since it can lyse the parasite *in vitro* with an IC_{50} value as low as 1,26 µM. DRS-SP4 and DRS-SP5 follow, with IC_{50} values of 2.31 µM and 19.06 µM respectively. Other researchers have characterized the effect of other antimicrobial peptides such as Tachyplesin-I, Clavanin-A, mytilin-A and Penaeidin-3, obtained from aquatic organisms, which displayed low antiparasitic effects against *T. cruzi* and *Leishmania braziliensis* (11). Only Tachyplesin-I presented antiparasitic activity at 12.5 µM concentration, while in our study, dermaseptins DRS-SP2 and DRS-SP4 displayed higher activity at lower concentrations (11).

It should be noted that in the aforementioned study and in most other research reports on the subject, the effect of the peptides or other chemicals is evaluated using epimastigotes, the vector-specific life stage of *T. cruzi* parasite (11,12,26,32–34). We have studied the effects effect of dermaseptins over trypomatigotes and amastigotes, which are mammalian-specific life cycle stages and therefore, relevant for human infection.

Our results show that dermaseptins reduce the number of intracellular parasites developing in the mammalian host-cell cytoplasm. DRS-SP2 had the best effect, with an IC_{50} of 5.855 µM, followed by DRS-SP5 with 14.59 µM and DRS-SP4 with 28.07 µM. The results of the colorimetric assays are supported by our microscopic studies with Giemsa-stained cells, which showed that the number of intracellular parasites decreases in the cells treated with DRS-SP2 and DRS-SP5. At the same time, few intracellular parasites were visible at 72 hours post infection while abundant intracellular parasites were visible in the control cultures.

Our electronic microscopy analyses evidenced the lytic effect of the three dermaseptins over *T. cruzi* trypomastigotes. Images suggest dermaseptins SP2 and PP5 have cytolytic over trypomastigotes, and they also seem to cause flagellum shedding. Conversely, SP4 seems to cause only flagellar alterations. Although additional testing is warranted, similar results were described by Camila et al. (35), who analyzed the effect of AMP Melittin, obtained from *Apis melifera* bee-venom, against *T. cruzi* via electronic microscopy. These researchers found the parasites presented abnormal conformations of the cellular body and with altered flagellar morphologies (35,36).

Among the mechanisms of action attributed to AMPs is the electrostatic interaction between the positive charge of peptides with the negative net charges of the cell membrane of the microorganisms causing their lysis (37–40). According to Proaño et al. (23), the dermaseptins analyzed in this study present a high net charge and are hydrophobic, and they have a bactericidal activity because they act on the bacterial membrane. Conversely, the yeast cell structure is different, and the mechanism of action underlying the dermaseptin antifungal activity could involve binding to the cell wall; inhibiting of DNA, RNA and protein synthesis; induction of signaling cascades or metabolic pathways; and production of free radical s(15,17,23,41). Electron microscopy results corroborate that the activity of dermaseptins is clearly lytic (42). Our results are consistent with such a mechanism of action for the studied peptides; however, additional studies are required.

The cytotoxic and anti-*T. cruzi* activity ratios of DRS-SP2, DRS-SP4 and DRS-SP5 are 7.80, 14.17 and 5.26, respectively, indicating that the peptides are much more lytic towards the trypomastigotes than to mammalian cells. Comparatively, these rations indicate the studied DRSPs are more efficient than other peptides reported in the literature at lysing *T. cruzi*. For example Penaeidin-3 obtained from *Litopenaeus vannamei*, has been reported to be only 4 times more

effective lysing the parasite than erythrocytes (11). Melittin, peptide obtained from *Apis mellifera* venom, was shown to be 100% effective on LLcMK2 cells at a concentration of 1,756 µM at 48 hours (35), while the dermaseptins DRS-SP2, DRS-SP4 and DRS-SP5 lost their cytotoxic effect progressively after 24 hours of treatment.

Conclusions

We show that DRS-SP2, DRS-SP4 and DRS-SP5 are capable of lysing both extracellular trypomastigotes and intracellular amastigotes of *T. cruzi.* Although these DRSs also display lytic activity against mammalian cells, parasites were more sensitive to them, and parasite/host cell cytotoxicity ratio ranged from 7.80 for DRS-SP2 to 14.14 for DRS-SP4. The lytic activity against *T. cruzi* is stronger than that recorded for other bioactive peptides, such as the DRS-SP2 that at 1.2 µM can kill the 50% of the parasites.

Acknowledgments

We are grateful to Andrea Vela, Ailín Blasco and Michelle Del Salto for their help in developing this research project. To the Pontifical Catholic University of Ecuador for providing funding for the development of this research (project QINV0209-IINV529010100) and likewise to the University of the Armed Forces (ESPE) for the assistance with the electron microscopy images.

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