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Research Article

RESEARCH ARTICLE

Cruzioseptins, antibacterial peptides from *Cruziohyla calcarifer* skin, as promising leishmanicidal agents

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One sentence summary: Peptides from frog skin secretion show antileishmanial activity.

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ABSTRACT

Screenings of natural products have significantly contributed to the discovery of novel leishmanicidal agents. In this study, three known cruzioseptins—antibacterial peptides from *Cruziohyla calcarifer* skin—were synthesized and evaluated against promastigotes and amastigotes stages of *Leishmania (L.) amazonensis* and *L. (V.) braziliensis*. EC50 ranged from 9.17 to 74.82 μM, being cruzioseptin-1 the most active and selective compound, with selectivity index $>$ 10 for both promastigotes and amastigotes of *L. (V.) braziliensis*. *In vitro* infections incubated with cruzioseptins at 50 μM showed up to ∼86% reduction in the amastigote number. Cruzioseptins were able to destabilize the parasite's cell membrane, allowing the incorporation of a DNA-fluorescent dye. Our data also demonstrated that hydrophobicity and charge appear to be advantageous features for enhancing parasiticidal activity. Antimicrobial cruzioseptins are suitable candidates and alternative molecules that deserve further *in vivo* investigation focusing on the development of novel antileishmanial therapies.

Keywords: cruzioseptins; frog skin secretion; leishmanicidal; peptides

INTRODUCTION

Leishmaniasis is a vector-borne tropical disease that affects mainly populations in less socioeconomically favored conditions (Charlton *et al.* 2018). As a result, leishmaniasis continues largely invisible to the global community. This disease is caused by intracellular protozoan parasites that belong to the *Leishmania* genus and about 95% of the cases occur in seven countries, including Brazil (Burza, Croft and Boelaert 2018).

Currently, leishmaniasis chemotherapy is based on the utilization of pentavalent antimonials, amphotericin B, paromomycin, pentamidine or miltefosine in different areas of the world (Frezard, Demicheli and Ribeiro 2009; Sunyoto, Potet and Boelaert 2018). However, this strategy presents several limitations in clinical practice, such as drug toxicity, high cost prolonged treatment, parenteral administration and selection of antimonial-resistant strains of *Leishmania*. In this scenario, the discovery of new leishmanicidal agents and molecular targets is key for the design of novel and improved therapeutic options (Alcântara *et al.* 2018).

For decades, the screening of natural products and the process of drug repositioning have played a fundamental role in the development of antileishmanial drugs (Le Pape 2008). The former has revealed new bioactive chemical structures with high activity from plants and animals, while the latter showed

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that some molecules previously characterized may be alternatives and useful for the treatment of this parasitic disease. In fact, many drugs available for treatment of leishmaniasis were not initially developed for this purpose (Charlton *et al.* 2018; Fernández-Prada *et al.* 2019). To illustrate this point, it is important to highlight that miltefosine, amphotericin B and paromomycin were originally designed as biomedical approaches to treat cancer, fungal and bacterial infections, respectively (Charlton *et al.* 2018).

Animal secretions enclose natural molecules that could be the basis for modern drugs and research tools (Simões-Silva *et al.* 2018; Almeida *et al.* 2019). Amphibian skins are composed by a number of diversified compounds, such as proteins, peptides, alkaloids and steroids, with multiple applications (Demori *et al.* 2019). In fact, the biomolecular diversity obtained from these secretions have shown promising antibacterial, antiviral, hypoglycemic, analgesic, anticancer and antiparasitic effects, including antileishmanial activity (Gomes *et al.* 2007). Particularly, peptides have been recognized as potential scaffolds for the development of novel agents for leishmaniasis treatment (Brand *et al.* 2013; Renata *et al.* 2017; Giovati *et al.* 2018; Mendes *et al.* 2019).

Proteomic, genomic and functional studies have investigated the chemical and biological diversity of peptides from amphibian secretions (Ma *et al.* 2010; Proaño-Bolaños *et al.* 2016; Mechkarska *et al.* 2018). Specifically, Proaño-Bolaños and co-authors characterized 15 peptides from *Cruziohyla calcarifer* (splendid leaf frog) skin secretion that had unique structural features, and as so were classified as part of a new family of antimicrobial peptides named cruzioseptins (Proaño-Bolaños *et al.* 2016). These peptides are cationic amphipathic compounds of 21–23 residues in length having low hemolytic activity and high antibacterial activity. Among the cruzioseptins evaluated, CSZ-1, which has greater hydrophobicity when compared to the others, was the most active, with a MIC of 3.77 μM against Gram-positive bacteria (Proaño-Bolaños *et al.* 2016).

Herein, we evaluated the antiprotozoal activity of three synthetic cruzioseptins against promastigotes and amastigotes forms of *L. (L.) amazonensis* and *L. (V.) braziliensis*, two important species that cause cutaneous forms of leishmaniasis in the New World. Insights on the mechanism of action regarding peptides' membranolytic effects are also presented.

MATERIAL AND METHODS

Peptide synthesis, purification and characterization

A total of three C-terminally amidated peptides belonging to cruzioseptins family (CZS-1: GFLDIVKGVGKVALGAVSKLF-NH2, CZS-2: GFLDVIKHVGKAALGVVTHLINQ-NH2 and CZS-3: GFLDVVKHIGKAALGAVTHLINQ-NH2) were synthetized automatically by the Fmoc/t-butyl approach using an automated microwave peptide synthesizer (Liberty Blue; CEM Biosciences, Matthews, NC). Briefly, by using a cleavage cocktail, the amino acid side chain protecting groups and peptides were simultaneously deprotected and removed from the resin, respectively. The cruzioseptins were purified using a reverse phase high-pressure liquid chromatography (RP-HPLC; Prep 150 LC System; Waters Corp., Milford, MA, USA) and further confirmed via matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy (MALDI-TOF MS Axima Confindence, Shimadzu Corp. Japan), as previously described by Proaño-Bolaños *et al.* (2016). All peptides used in this study were >95% pure. Stock solutions were prepared at 2 mM in phosphate buffered saline (PBS 1X; Sigma-Aldrich, Saint Louis, MO, USA, Merck KGaA, Darmstadt, Germany) and stored at −20°C until use.

In silico physicochemical characterization

To investigate the structure–activity relationships, the basic physicochemical properties of synthetic cruzioseptins were analyzed by bioinformatic tools. The molecular mass was calculated by on line Peptide Mass Calculator v3.2 software. The charge (pH = 7.0) and pI were determined using the PepDraw tool (http://www.tulane.edu/~{}biochem/WW/PepDraw/). The grand average of hydropathy (GRAVY) value for each peptide sequence was estimated by GRAVY CALCULATOR (http://www.gravy-calculator.de/). The PEPTIDE CALCULATOR software was used to determine the ratio of hydrophilic residues/total number of residues. Additionally, the primary structures were compared using the Kalign multiple sequence alignment algorithm available in Expasy server (Madeira *et al.* 2019).

Parasites

Two *Leishmania* reference species were used in this study: *Leishmania (Leishmania) amazonensis* (IFLA/BR67/PH8) and *Leishmania (Viannia) braziliensis* (MHOM/BR/75/M2903). The parasites were routinely cultured as promastigotes at 25°C in medium 199 (Sigma-Aldrich, Merck KGaA) supplemented with 10% heat-inactivated fetal bovine serum, 10 mM adenine, 4 mM biotin, 1 M HEPES, 5 mM L-glutamine, 6 μg/mL hemin and penicillin (100 U/mL)/streptomycin (100 μg/mL; Amresco Inc., West Chester, PA, USA; complete medium 199). *L. (V.) braziliensis* cultures were supplemented with 2% human male sterile urine. Amastigotes were isolated and purified from BALB/c mice as previously described by Mendes *et al.* (2019).

Activity of cruzioseptins against promastigotes, lesion-derived amastigotes and macrophages

In vitro activity of cruzioseptins was initially evaluated against the promastigote stage. Parasites harvested in the early-logarithmic growth phase (5×10^6/mL) were seeded in a 96-well microtiter plate containing 200 μL of complete medium 199. After, cruzioseptins were added at final concentration of 0, 12.5, 25, 50, 75 and 100 μM and incubated for 24 h at 25°C. *Leishmania*'s viability was measured according to the reduction of cell metabolic activity using the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromid) colorimetric assay as described by Miguel *et al.* 2007. Briefly, stock solutions of the MTT reagent (Sigma-Aldrich, Merck KGaA) were prepared in PBS 1X at 5 mg/mL and kept at 4°C for up to 10 days. A total of 30 μL of MTT solution was added to each plate well and incubated for 2 h at 25°C. Next, cell suspensions were lysed with 20 μL of 20% sodium dodecyl sulphate (Sigma-Aldrich, Merck KGaA) for absorbance reading using the AgileReader Elisa Plate Reader (AvansBio, Avans Biotechnology, Taiwan). 50% effective concentration (EC50) of each cruzioseptin was determined using a non-linear regression curve (software OriginLab 8.0), as previously reported by Craig *et al.* (2017).

The activity of cruzioseptins against the amastigote stage was firstly investigated using free amastigotes derived from mouse cutaneous lesions. In this case, an amastigote to promastigote differentiation assay was conducted with 1.6×10^6 parasites/mL incubated in 96 well-plate (Conrning, Inc., New York, USA) containing 200 μL of complete medium 199 and cruzioseptins at 0, 3.12, 12.5, 25, 50 and 100 μM for 72 h, 25°C. The number of differentiated promastigotes was estimated using a hemocytometer. Subsequent determination of the EC50 values was performed as described above.

In order to assess the toxicity and intramacrophagic antileishmanial effect of the peptides, bone marrow-derived macrophages (BMDMs) were obtained as previously described by Miguel *et al.* (2013). In brief, mice femur and tibia bones were isolated and precursor cells were flushed out from the bones lumen and recovered with 5.0 mL of RPMI 1640 (Sigma-Aldrich, Merck KGaA) medium containing 20% of L-929 fibroblasts culture supernatant, 20% fetal bovine serum, penicillin (100 U/mL) and streptomycin (100 μg/mL). BMDMs were cultured in Petri dishes (Conrning, Inc.) for 7 days at 37°C and 5% CO_2. Fresh RPMI 1640 medium was added after the third day of culture. *In vitro* peptide toxicity towards host cells was determined by MTT cell viability (Miguel *et al.* 2007). BMDMs (5×10^5 cells/mL) were cultured into 96-well plates with 200 μL of medium RPMI 1640 for 3 h at 37°C and 5% CO_2. After cell adhesion, BMDMs were incubated with several concentrations of cruzioseptins (3.125–150 μM) or in its absence (untreated control cells) for 24 h at 37°C and 5% CO_2. The 50% cytotoxic concentration (CC50) was defined as the peptide concentration that inhibited 50% of BMDMs' growth when compared to untreated control cells. Pure DMSO-diluted amphotericin B (Sigma-Aldrich, Merck KGaA) was used for positive control of parasite killing as described (Miguel *et al.* 2007).

Moreover, the selectivity index (SI) was determined by dividing the BMDMs CC50 value by the EC50 value for *L. (L.) amazonensis* and *L. (V.) braziliensis* promastigotes and lesion-derived amastigotes.

In vitro infections

Intramacrophagic leishmanicidal effect was performed by incubating *Leishmania*-infected cells with different concentrations of cruzioseptins according to Parra *et al.* (2018). Briefly, 5×10^5 BMDMs/mL were cultured in 24-well plates with coverslips containing 500 μL RPMI 1640 medium for 3 h at 37°C and 5% CO_2. Adherent BMDMs were infected with stationary-phase promastigotes of *Leishmania* in a 1:10 ratio for 4 h at 34°C in a 5% CO_2 atmosphere. The excess of non-internalized promastigotes was removed by washing with warm PBS 1X. Afterwards, cruzioseptins were added at 0, 3.125, 12.5 and 50 μM and incubated for 24 h for at 34°C and 5% CO_2. Infections were washed with PBS 1X, fixed and stained using the Instant Prov kit (Newprov, Pinhais, Brazil). The number of intracellular parasites was determined by counting 300 BMDMs in three coverslips for each condition, from randomly chosen microscopic fields (1000× magnification; Leica LAS Core microscope system, Germany). The Ethics Committee on Animal Use of the University of Campinas (CEUA-UNICAMP) approved the experiments using infected animals (#3484–1 and #4951–1).

Fluorimetric evaluation of promastigote membrane permeability

The protocol for plasma membrane permeabilization was carried out according to Cohen *et al.* (1990). Approximately 10^7 cells/mL were resuspended in a buffer solution (10 mM $C_6H_{12}O_6$, 11 mM KCl, 140 mM NaCl and 75 mM Tris-HCl, pH 7.5). After the baseline was established, 10 μM ethidium bromide (EB; Sigma-Aldrich, Merck KGaA) was added and after 1 min, the volume equivalent to the EC50 of each synthetic cruzioseptin was added. Changes in permeability plasma membrane as a function of time were monitored continuously using a fluorescence spectrophotometer (Hitachi F-2500, Japan) at 37°C (excitation = 590 nm, emission = 560 nm). Maximal fluorescence was achieved after the addition of 100 mM digitonin.

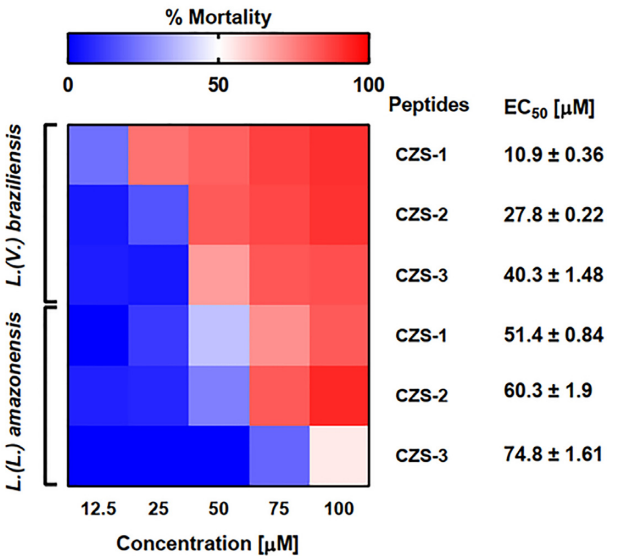

Figure 1. Heat map of the *in vitro* effect of cruzioseptins on *Leishmania* spp. promastigotes. Promastigotes (5×10^6/mL) of *L. (V). braziliensis* and *L. (L.) amazonensis* were treated with different concentrations of cruzioseptins for 24 h at 25°C. The color scale bar for the mortality percentage is shown at the top. The colors represent relative high (red) and low (blue) cell mortality. EC50 ± standard deviation values are shown on the right column. Three independent experiments were performed in triplicate.

Statistical analysis

Results are presented as mean ± standard deviation. One-way analysis of variance (ANOVA) with Tukey's Honest Significant Difference test were used for statistical analysis by using Prism 8 software (GraphPad Software, San Diego, CA). Differences at $P < 0.05$ were considered significant.

RESULTS

In silico properties of cruzioseptins 1, 2 and 3

The three amidated peptides evaluated in this study share diverse biochemical properties (Table 1). Briefly, these peptides have similar length (21-23 aa), isoelectric points (10.65–10.89) and percentage of hydrophilic residues (22-24%). CZS-2 and CZS-3 have a charge of +2, while CZS-1 has a net charge of +3. The sequence similarity analysis of CZS-2 and CZS-3 revealed a high degree of shared amino acid identity (86.96%). Additionally, CZS-1 shares 66.67% and 61.90% identity with CZS-3 and CZS-2, respectively. The main difference between the three cruzioseptins is their hydrophobicity, expressed here by the GRAVY value. CZS-1 has a higher score indicating greater hydrophobicity.

Activity of cruzioseptins against *Leishmania* spp

In the first round of *in vitro* experiments, the leishmanicidal activity of synthetic peptides was determined against the promastigote forms. The parasite viability assay showed that the ability of MTT reduction to formazan was significantly affected by the exposure to different concentrations of the three cruzioseptins in a dose-dependent manner (Fig. 1). In general, CZS-1, -2 and -3 presented a parasiticidal effect on promastigote forms of *L. (L.) amazonensis* and *L. (V) braziliensis* after 24 h in a micromolar range. *L. (V.) braziliensis* was more susceptible to

Table 1. Cruzioseptins biochemical features. The peptides share diverse properties and also amino acid sequence similarity. CZS-1 is the most cationic and hydrophobic peptide.

Peptides	Length	Theoretical mass (Da)	pI	Charge	GRAVY*	Hydrophilic/total residues**	Percent Identity Matrix (%)	
							CZS-1	CZS-2
CZS-1	21 aa	2117.60	10.89	+3	1.157	24%	100	61.90
CZS-2	23 aa	2428.90	10.65	+2	0.739	22%	61.90	100
CZS-3	23 aa	2400.85	10.65	+2	0.635	22%	66.67	86.96

*Grand average of hydropathicity.
**Ratio of hydrophilic residues/total number of residues.

the action of cruzioseptins, as evidenced by the calculated EC50 and lowest survival rate after incubation. CZS-1 showed greater antipromastigote activity for both species (EC50: 10.9 ± 0.36 and 51.4 ± 0.84 μM for *L. (V.) braziliensis* and *L. (L.) amazonensis*, respectively).

Cruzioseptins showed activity against lesion-isolated amastigotes as well. In this case, amastigotes were allowed to differentiate into promastigotes, in the presence of each peptide, as a measurement of cell viability. A significant decrease in the number of viable parasites was observed (Fig. 2A). Similar to the antiprotozoal effects observed for continuously cultured promastigotes, cruzioseptins induced a greater decrease in the number of freshly differentiated promastigotes. Among the peptides, CSZ-1 showed to be more active with EC50 values of 9.17 ± 0.72 and 13.15 ± 1.93 against *L. (V.) braziliensis* and *L. (L.) amazonensis*, respectively (Fig. 2B and C). Furthermore, when EC50 values were compared, lesion-derived amastigotes were 1.2 to 3.9-fold more susceptible to cruzioseptins than promastigotes.

Cytotoxicity of cruzioseptins on bone marrow-derived macrophages (BMDMs)

The toxicity of the peptides against BALB/c BMDMs was evaluated after peptide incubation in a 2-fold serial dilution from 150 to 1.85 μM. CC50 values varied from 112.34 μM (CSZ-1) to 127.19 μM (CZS-3). The experimental results (CC50) in BMDMs showed values greater than all the EC50 determined for the promastigote and amastigote stages of both species. Selectivity index (SI) was estimated and CZS-1 showed SIs >10 for both promastigotes and amastigotes of *L. (V.) braziliensis* (Table 2).

Cruzioseptins reduces intracellular parasite burden

After assessing the activity of the peptides against axenic parasites, we investigated their effect in infected BMDMs. All peptides led to the reduction of the parasite burden in a dose-dependent manner, where CZS-1 was more effective than CZS-2 and CZS-3 ($P < 0.05$). Again, *L. (V.) braziliensis* was more sensitive to CZS-1, CZS-2 and CZS-3 than *L. (L.) amazonensis*, being more potent at 50 μM (86.6, 80.9 and 69.9% of amastigote number reduction in relation to untreated infections, respectively). Examples of photomicrographs illustrate the decrease of *L. (V.) braziliensis* and *L. (L.) amazonensis* amastigotes within BMDMs for CZS-1 at 50 μM (Fig. 3C). As expected, parallel assays included amphotericin B as positive controls for parasite killing, showing EC50 in the lower micromolar range of 0.07 ± 0.84 and 0.09 ± 0.34 μM for intracellular *L. (V.) braziliensis* and *L. (L.) amazonensis* amastigotes, respectively.

Plasma membrane permeabilization induced by cruzioseptins

Our next step was to investigate whether these peptides could disturb parasite's plasma membrane integrity. In this case, promastigotes of *L. (V.) braziliensis* were incubated with ethidium bromide (EB) and each cruzioseptin at their EC50 in buffer solution for fluorescence detection (Fig. 4). Rapid EB incorporation for all the peptides was detected; with a slightly less marked permeability for CZS-3. No differences were detected among the peptides; a trend also observed after the addition of digitonin, a mild nonionic detergent used to allow the maximum incorporation of EB.

DISCUSSION

Antibacterial peptides with leishmanicidal properties have been characterized from a wide range of natural sources (Luque-Ortega and Rivas 2010; Marr, McGwire and McMaster 2012), including frog skin secretions (Mangoni *et al.* 2005; Renata *et al.* 2017). These samples contain different peptides and proteins, which can act as drug scaffolds with different biomedical applications (Proaño-Bolaños *et al.* 2019). Moreover, in recent years, peptide investigation has been increasingly proposed as a valuable shortcut for new leishmaniasis treatment options (Raja *et al.* 2017; Zahedifard and Rafati 2018). There is a significant difference between the numbers of characterized peptides from frogs tested against bacteria *versus* eukaryotic cells (Guerrero *et al.* 2004; Zampa *et al.* 2009). Many studies focus on bacteria, as the recent work by Proaño-Bolaños *et al.* (2019) that identified several antimicrobial peptides from *Agalychnis spurrelli*. Taking into account that the peculiar biochemical characteristics of antibacterial peptides have helped in the design of new leishmanicidal agents (Cobb and Denny 2010; McGwire and Kulkarni 2010), in the present work we investigated cruzioseptins' activity against *Leishmania* species that cause distinct forms of cutaneous leishmaniasis in South America.

Similar to other antibacterial peptides isolated from skin frog secretions, such as dermaseptin (Brand *et al.* 2013) and temporin (Abbassi *et al.* 2013), the cruzioseptins reported here have an antipromastigote and antiamastigote activity in the micromolar range. Interestingly, amastigotes were more sensitive to the peptides than promastigotes (Figs 1 and 2B, C). In a similar manner, melittin (Pereira *et al.* 2016), bombinins H2 and H4 (Mangoni *et al.* 2006) and 4-amino acid peptide KDEL (Cao *et al.* 2019) were more active against the intracellular stage. These data are relevant for drug screenings, as the amastigote is the clinically relevant stage of parasite (Alcântara *et al.* 2018).

In general, CZS-1 showed the lowest EC50 values when compared to CZS-2 and CZS-3. Considering the primary structures

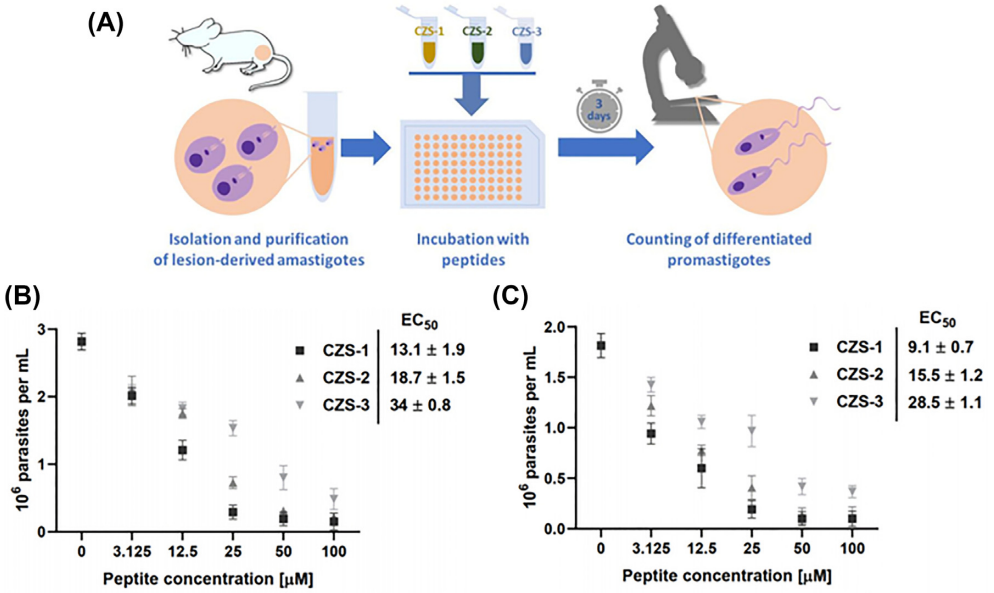

Figure 2. *In vitro* effect of cruzioseptins on promastigote differentiated from lesion-derived amastigotes. **(A)** Schematic overview of the amastigote to promastigote differentiation assay. Promastigotes were obtained by *in vitro* conversion of amastigotes at 25°C in complete medium 199 after 72 h. Amastigotes of **(B)** *L. (V.) braziliensis* and **(C)** *L. (L.) amazonensis* were recovered from BALB/c mice lesion sites and cultured for 72 h in the absence or in the presence of the peptides CZS-1–3. The viability of amastigotes was measured by assessing the number of motile promastigotes using a hemocytometer for each condition. A total of two independent experiments were performed in triplicates. EC50 ± standard deviation values are presented for each peptide in (B) and (C).

Table 2. Cruzioseptins' cytotoxicity against murine BMDMs and selectivity index. The viability of BMDMs was measured after exposure to different concentrations of cruzioseptins. Cytotoxic concentration for 50% of macrophagic cultures (CC50) was expressed in the micromolar range (± standard deviation). The selectivity index (SI) represents the ratio between the CC50 and EC50 values for promastigotes and amastigotes upon incubation with CZS-1, -2 and -3.

		Promastigotes		Lesion-isolated amastigotes	
Peptides	CC50 (μM) ± SD	*L. braziliensis*	*L. amazonensis*	*L. braziliensis*	*L. amazonensis*
CZS-1	112.34 ± 0.2	10.28	2.18	12.25	8.54
CZS-2	119.82 ± 1.4	4.29	1.98	7.72	6.39
CZS-3	127.19 ± 0.3	3.15	1.69	4.46	3.54

and physicochemical characteristics of them, these data indicated that the contribution of hydrophobicity to their leishmanicidal activity must be taken into account (Table 1). This characteristic is supported by a previous study with magainins from the African clawed frog (Guerrero *et al.* 2004). Furthermore, it has been suggested that cationicity is also determinant for antiprotozoal activity (Rivas, Luque-Ortega and Andreu 2009). Feder, Dagan and Mor (2000) reported that a dual amino acid substitution in dermaseptin-S4 enhanced its effect against *L. major*. Among the investigated cruzioseptins, CZS-1 has a higher net load (+3), and showed higher activity against intracellular and extracellular forms of *Leishmania* spp in culture. CZS-2 and CZS-3 were less potent (higher EC50), probably due to a lower presence of residues with positive side chains. On other hand, CZS-2 and CZS-3 have the same charge (+2), but did not show the same magnitude of antileishmanial effect, as observed in EC50 determined by *in vitro* assays. Minimum differences in the hydrophobicity profiles of the peptides could explain the variation in their efficacy. This observation highlights the requirement for a hydrophobic character to interact with the parasite's membranes, as previously suggested (Khalili *et al.* 2019). In fact, with respect to the antileishmanial activity against promastigotes, the uptake of EB and fluorescence increase upon incubation with the peptides point to a membrane disruption mode of action (Fig. 4). Our findings are in agreement with several studies using different membrane-damaging peptides, such as: cecropin A-aelittin hybrid peptides (Diaz-Achirica *et al.* 1998), temporins (Mangoni *et al.* 2005), brevinin (Zahedifard *et al.* 2019), bombinins (Mangoni *et al.* 2006) and 13-mer peptides based on phospholipase and oligoarginine (Mendes *et al.* 2019).

Investigation of a given compound's toxicity to macrophages and its ability to prevent the survival of intracellular amastigotes are key for finding potential leishmanicidal candidates (Alcântara *et al.* 2018). Regarding cruzioseptins' cytotoxicity, CC50 values varied between 112.3 and 127.2 μM, which are higher than the EC50 values determined for both forms of *Leishmania* spp, leading to good selectivity indexes, particularly for CZS-1 against *L. (V.) braziliensis* (Table 2). Infection images presented in Fig. 3C (b and d) also point to the maintenance of macrophages integrity after incubation with CZS-1 at 50 μM. Results with this peptide are similar or superior to further data considering leishmanicidal peptides, such as andropin (Perez-Cordero *et al.* 2011) and melittin (Pereira *et al.* 2016). The selectivity of peptides with dual antileishmanial and antibacterial

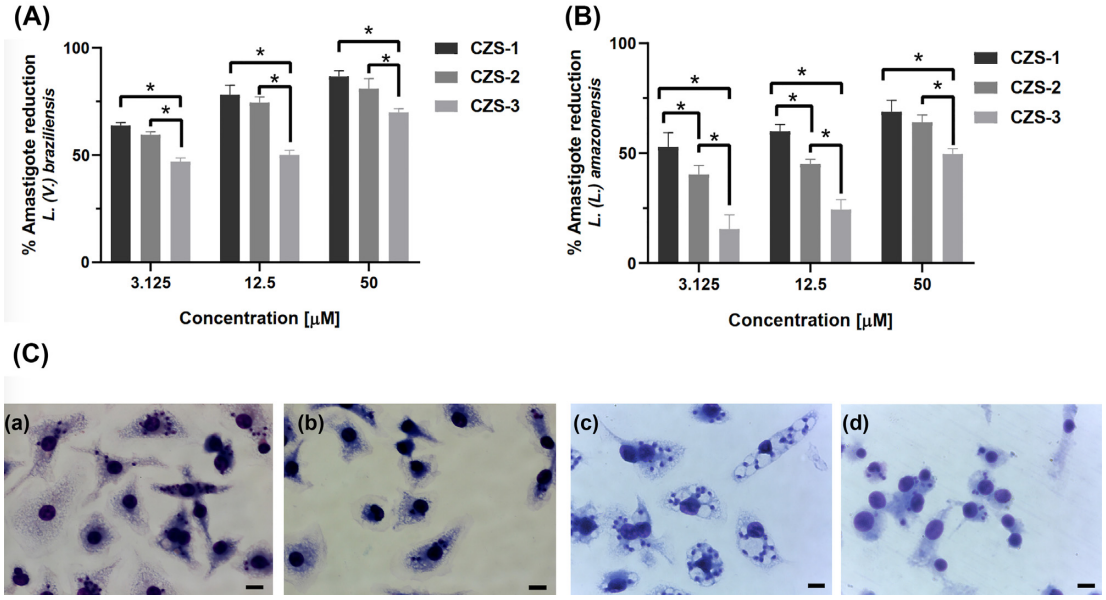

Figure 3. Evaluation of cruzioseptins' effect on intracellular amastigotes using a macrophage infection model. A significant decrease of amastigote intramacrophage survival was observed by incubation with peptides. The data were expressed as the mean ± SD and were analyzed by one-way ANOVA with Tukey's post hoc test (*$P < 0.05$). BMDMs were infected with **(A)** *L. (V.) braziliensis* and **(B)** *L. (L.) amazonensis*. **(C)**: Representative images of untreated *L. (V.) braziliensis* (a) and *L. (L.) amazonensis* (c) infected BMDMs. It is possible to visualize a significant decrease in *L. (V.) braziliensis* (b) and *L. (L.) amazonensis* (d) intracellular parasites after incubation with CZS-1 at 50 μM. Scale bar = 5 μm. A total of two independent experiments were performed in triplicates.

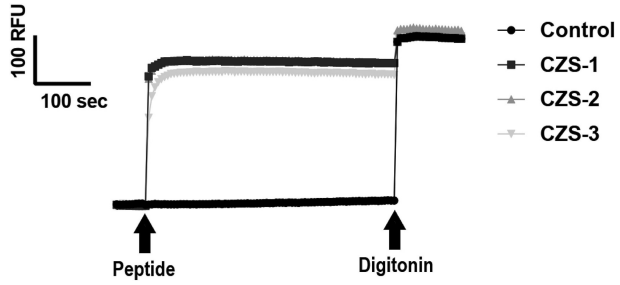

Figure 4. Incorporation of DNA-binding fluorescent dye by promastigotes of *L. (V.) braziliensis* upon cruzioseptins incubation. Peptides at their EC50 were added to 10^7 cells/mL *Leishmania* suspensions. Changes on permeability parasite membrane were monitored during 600 s. The dye uptake was quantified by fluorescence spectroscopy (590 nm excitation, 560 nm emission). Digitonin was used to maximize the cell permeability to EB and fluorescence signal. Arrows indicate the addition of peptides (EC50) or digitonin.

activities and low toxicity for macrophages has been reported for other synthetic molecules from frog skin secretions (Brand *et al.* 2013; Pinto *et al.* 2013).

Leishmania-infected BMDMs treated with three cruzioseptins presented a significant decrease in intramacrophage survival of amastigotes (Fig. 3A and B). Less pronounced reductions in the number of intracellular amastigotes were observed for infected cells incubated with CZS-3. In contrast, CZS-1 and CZS-2 showed a similar reduction in intramacrophage parasite burden; unlike the higher CZS-1 leishmanicidal activity found axenic parasites. Earlier studies with other synthetic peptides have already addressed to these differences regarding the potency of leishmanicidal activity in cell culture and macrophage infection assays (Perez-Cordero *et al.* 2011; Marr *et al.* 2016).

The intracellular nature of amastigote creates additional barriers and challenges for the leishmanicidal activity of peptides (Sundar and Singh 2018). In addition to the ability of cruzioseptins to disrupt surface-membrane of parasites, other intramacrophagic and immune response mechanisms that interfere with key processes for the amastigote survival can be triggered by the peptides and remain to be explored. Some works have demonstrated the capacity of mammalian antimicrobial peptides to promote the pro- and anti-inflammatory cytokine release and to regulate the host's response to infection, such as cutaneous *Leishmania* infection (Bowdish *et al.* 2005; Kulkarni *et al.* 2011; Erfe *et al.* 2012). In parallel, dermaseptin (Perez-Cordero *et al.* 2011), human neutrophil peptide-1 (Dabirian *et al.* 2013), melittin (Pereira *et al.* 2016) and crotamin (Katz *et al.* 2020) appear to exert their leishmanicidal effects through mechanisms that involve macrophage activation.

CONCLUSIONS

CZS-1, CZS-2 and CZS-3 were able to decrease the viability of extracellular and intracellular *Leishmania* spp. *in vitro* in a dose-dependent manner. These bioactive compounds significantly reduced intramacrophagic parasite infections. The peptide hydrophobicity appears to contribute to their antileishmanial action, which is related to membranolytic effects on promastigotes. Our results show that antibacterial cruzioseptins, mainly CZS-1 and CZS-2, deserve further investigation as promising peptide lead structures for generation of new drugs for the treatment of leishmaniasis.

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Conflicts of Interest. None declared.

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