Comparative Biochemistry and Physiology, Part C 226 (2019) 108612

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part C

journal homepage: www.elsevier.com/locate/cbpc

Potential use of 13-mer peptides based on phospholipase and oligoarginine as leishmanicidal agents

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ARTICLE INFO

Keywords:
Leishmanicidal activity
Oligoarginine
Peptide
Phospholipase

ABSTRACT

Phospholipase A_2 toxins present in snake venoms interact with biological membranes and serve as structural models for the design of small peptides with anticancer, antibacterial and antiparasitic properties. Oligoarginine peptides are capable of increasing cell membrane permeability (cell penetrating peptides), and for this reason are interesting delivery systems for compounds of pharmacological interest. Inspired by these two families of bioactive molecules, we have synthesized two 13-mer peptides as potential antileishmanial leads gaining insights into structural features useful for the future design of more potent peptides. The peptides included p-Acl, reproducing a natural segment of a Lys49 PLA_2 from *Agkistrodon contortrix laticinctus* snake venom, and its p-AclR7 analogue where all seven lysine residues were replaced by arginines. Both peptides were active against promastigote and amastigote forms of *Leishmania* (*L.*) *amazonensis* and *L.* (*L.*) *infantum*, while displaying low cytotoxicity for primary murine macrophages. Spectrofluorimetric studies suggest that permeabilization of the parasite's cell membrane is the probable mechanism of action of these biomolecules. Relevantly, the engineered peptide p-AclR7 was more active in both life stages of *Leishmania* and induced higher rates of ethidium bromide incorporation than its native template p-Acl. Taken together, the results suggest that short peptides based on phospholipase toxins are potential scaffolds for development of antileishmanial candidates. Moreover, specific amino acid substitutions, such those herein employed, may enhance the antiparasitic action of these cationic peptides, encouraging their future biomedical applications.

1. Introduction

Leishmaniasis is one of the major vector-borne neglected tropical diseases, endemic in >100 countries, and particularly devastating for low to middle income countries (Marr et al., 2016; Burza et al., 2018). This alarming public health problem is caused by more than twenty *Leishmania* species, which are capable of infecting both insects and mammals (Marr et al., 2012). Statistics on the status of leishmaniasis reveal that approximately 20 million people are infected worldwide with ~30,000 annual deaths (Alvar et al., 2012). Clinical manifestations of leishmaniasis depend on parasite species, geographical localization and the immune status of the host, varying from localized cutaneous lesions to a systemic multiorgan infection or visceral form (Pigott et al., 2014; Alcantara et al., 2018). Available therapies have been limited by toxicity, leading to several adverse effects, and parasite resistance which, associated with the absence of vaccines, highlights the urgent need to develop new and more efficient approaches (Alcantara et al., 2018).

Phospholipases A_2 (PLA_2s) are versatile and ubiquitous biomolecules found in snake venoms (Almeida et al., 2016a; Almeida et al., 2017), that present a remarkable diversity of pharmacological effects, including antiparasitic activity against *Leishmania* species (Simoes-Silva et al., 2018). Several PLA_2s with *in vitro* and *in vivo* leishmanicidal action have been isolated and characterized from different venoms of

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https://doi.org/10.1016/j.cbpc.2019.108612
Received 19 July 2019; Received in revised form 21 August 2019; Accepted 22 August 2019
Available online 24 August 2019
1532-0456/

Crotalus durissus terrificus (Barros et al., 2015), *Bothrops pauloensis* (Nunes et al., 2013), *Bothrops moojeni* (Stabeli et al., 2006), *Bothrops mattogrossensis* (Moura et al., 2014), and *Bothrops brazili* (Costa et al., 2008). Recently, studies with liposomal formulation of PLA_2s have reinforced the interesting application of these snake toxins as antileishmanial agents (de Barros et al., 2018). Taking this into account, the primary structures of PLA_2s represent an important window of opportunity for new drug prototypes designed for leishmaniasis treatment.

Small cationic oligopeptides synthesized on the basis of the C-terminal region of PLA_2s have shown a high therapeutic potential similar to their parent proteins in a number of biological assays (Almeida et al., 2017; Almeida et al., 2018a; Almeida et al., 2018b). These molecules have been recognized primarily for their antitumor (Araya and Lomonte, 2007) and antimicrobial activity (Santos-Filho et al., 2015; Almeida et al., 2018b), although some studies have also revealed their antiparasitic potential (Costa et al., 2008). In this context, Costa et al. (2008) have reported the activity of 13-mer synthetic peptides derived from myotoxin phospholipases A_2 of *Bothrops brazili* against *L.* (*L.*) *amazonensis* and *L.* (*V.*) *braziliensis*.

Biomimetic peptides derived from PLA_2s are mainly characterized by their positive charge, presence of hydrophobic and conserved lysine residues (Lomonte et al., 2010; Corrêa et al., 2016). PLA_2s-derived peptides show similarity to cationic host defense peptides (AMP) and cell-penetrating peptides (CPP) (Almeida et al., 2018b). The physicochemical characteristics of CPP result in biological membrane penetration and for this reason they are extensively studied as vehicles for cellular delivery of distinct molecular loads (Takeuchi and Futaki, 2016). In contrast, AMP have been extensively studied for their antibacterial action (Bahar and Ren, 2013). Moreover, promising studies have determined the potential of these small molecules towards the control of *Leishmania* infection (Kulkarni et al., 2011; Marr et al., 2012), while; in addition, several bioactive peptides from different sources have showed dual activities (Cobb and Denny, 2010; Pérez-Cordero et al., 2011; Felício et al., 2017).

In the present work, two 13-mer cationic peptides were synthesized and evaluated for their antileishmanial potential: peptide p-Acl, corresponding to the natural sequence of the C-terminal region (115–129) of ACL, a Lys49 PLA_2 from the venom of the broad-banded copperhead snake *Agkistrodon contortrix laticinctus* (UniProtKB - P49121-PA2H1_AGKCL); and its analogue p-AclR7, where all native lysine residues were replaced by arginines, hence combining the natural characteristics of p-Acl with those of oligoarginines, well-known members of the CPP family. The antiparasitic potential of both peptides were evaluated against promastigotes and amastigotes of distinct *Leishmania* species. Cell membrane permeabilization was also investigated as a possible molecular mechanism capable of explaining the leishmanicidal effect of 13-mer peptides.

2. Material and methods

2.1. Peptide design and synthesis

13-mer peptides were chemically synthetized using Fmoc/^{t}Bu protocol on a CEM Liberty1 system for microwave-assisted peptide synthesis, as previously reported (Almeida et al., 2018a). Each peptide was prepared as C-terminal carboxamide and synthesized from the carboxyl to the amino terminus. Piperidine was used for removal of Fmoc protecting groups. Once the whole polypeptide chain was assembled, it was released from the resin (Rink Amide MBHA) with concomitant removal of side-chain protecting groups, by acidolysis with a cleavage cocktail composed of 90% trifluoroacetic acid, 2% of anisole 2%, 3% ethane-1,2-dithiol, and 5% of thioanisole 5%. Crude peptides were precipitated using cold diethyl ether, collected and dried in a desiccator, reconstituted in 10% aqueous acetic acid, lyophilized and stored at −20 °C freezer until further use.

2.2. Peptide purification

The purification and analytical analysis of synthetic peptides were performed using a reverse phase high-pressure liquid chromatography (RP-HPLC) Accela (Thermo Fisher Scientific) as previously described (Almeida et al., 2018a). Briefly, the elution was performed on a C18 Nucleodur gravity column (Macherey-Nagel, USA) 5 µm particle size and dimensions 4 mm ID x 125 mm over a gradient of 100% solvent A (100% H_2O with 0.1% (v/v) formic acid) to reach 100% solvent B (ACN with 0.1% (v/v) formic acid) at 30 min at a flow rate of 0.5 mL/min. The purity degree of peptide was determined at 220 nm. Purified peptides were lyophilized and stored at −20 °C. For functional assays, the peptides were resuspended in cell culture media.

2.3. Identification of synthetic peptides by mass spectrometry

The exact value $^m/_z$ of the synthetic peptides were determined by mass spectrometry on an LTQ OrbitrapTM XL hybrid mass spectrometer (Thermo Fischer Scientific, Bremen, Germany) controlled by LTQ Tune Plus 2.5.5 and Xcalibur 2.1.0. The electrospray ionization source settings and MS data handling were the same as those used in the Almeida et al. (2018a).

2.4. Determination of physico-chemical properties and bioinformatic analyzes

Predictions about physico-chemical parameters of 13-mer peptides synthetized and the multiple alignment analysis were performed using on line bioinformatic tools available in Expasy server, such as PepDraw (Tulane University, New Orleans, LA, USA; http://www.tulane.edu/~biochem/WW/PepDraw/index.html) and ClustalW (Larkin et al., 2007). The theoretical properties were calculated *in silico* according to the amino acid sequence of amide peptides.

2.5. Animals

Female BALB/c mice aged 4–8 weeks were obtained from CEMIB-UNICAMP (Centro Multidisciplinar para Investigação Biológica). Animals received standard diet and water *ad libitum*. The protocol for animal experimentation was approved by the Ethical Committee for Animal Experimentation of the Biology Institute - UNICAMP (#4951-1/2018).

2.6. Parasites

Promastigote forms of *L.* (*L.*) *amazonensis* (IFLA/BR67/PH8 and MHOM/BR/1973/M2269) strains and *L.* (*L.*) *infantum* (MHOM/BR/1972/LD) were cultured in Medium 199 (Sigma-Aldrich) supplemented with 20% heat-inactivated fetal bovine serum (FBS) (Sigma-Aldrich), 10 mM adenine, 5 mM L-glutamine and penicillin (100 U/mL) and streptomycin (100 µg/mL) (Sigma, USA), at 26 °C (Miguel et al., 2013). *L.* (*L.*) *infantum* cultures were supplemented with 5% human male sterile urine. For this study, fresh amastigotes of *L.* (*L.*) *amazonensis* (*PH8*) were obtained from BALB/c mice non-necrotic cutaneous lesions infected at the basis of the tail for 30 days. To isolate the amastigotes, lesion site tissues were excised and transferred to a glass dounce homogenizer in 10 mL cold phosphate buffered saline 1× (PBS 1×), and centrifuged at 100 ×*g* for 10 min at 4 °C. The supernatant was then collected and centrifuged in 5 mL PBS 1× at 800 ×*g* for 10 min, 4 °C. After the first wash, the pellet was resuspended in 5 mL buffer and washed 3 times. Amastigotes were observed under the light microscope and maintained in Medium 199, pH 4.8, supplemented with 20% FBS, penicillin (100 U/mL) and streptomycin (100 µg/mL) for 24 h at 32 °C.

2.7. Evaluation of leishmanicidal activity

2.7.1. Effect of synthetic peptides on promastigotes

A total of 5×10^6/mL early-logarithmic-phase promastigotes of *Leishmania* spp. was placed into a 96-well plate in Medium 199. Parasites were incubated in the presence of different 13-mer peptide concentrations (0–250 μM) at 27 °C for 24 h. Control groups were consisted of promastigotes in and medium alone. Promastigotes viability was assessed by the MTT (3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium) colorimetric assay (Parra et al., 2018). The effective concentration of 50% (EC_{50}) was calculated using the statistical package of GraphPad Prism. All experiments were performed in triplicate from three independent experiments and data were expressed as mean ± standard deviation (± SD).

2.7.2. Effect of synthetic peptides on amastigotes

L. (*L*) *amazonensis PH8* amastigotes (5×10^5/mL in amastigote media) were dispensed into a 96-well plate and treated with the synthetic peptides (0–100 μM) for 24 h at 32 °C. Control groups were consisted of amastigotes in and medium alone Parasite viability was then measured using MTT assay (Parra et al., 2018). The 50% effective concentration (EC_{50} value) against amastigotes was determined as described above.

2.8. Differentiation of bone marrow-derived macrophages (BMDMs)

Bone marrow-derived macrophages were obtained as previously described by Miguel et al. (2013) with some modifications. BMDMs were obtained from both femur and tibia of individual Balb/C mice. After flushing bone marrow contents out with 5.0 mL of RPMI 1640 medium (Sigma-Aldrich) in the presence of growth factors (20% of L-929 fibroblasts culture supernatant and 20% fetal bovine serum) samples were plated in Plastic sterile Petri dishes containing penicillin (100 U/mL) and streptomycin (100 μg/mL) (Sigma, USA). Petri dishes were incubated for 7 days at 37 °C and 5% CO_2. On the 3rd day, 5.0 mL of complete RPMI (20% L-929 and 20% FBS) was added to each plate.

2.8.1. Effect of synthetic peptides on Leishmania infection

The effect of 13-mer peptides was evaluated in *Leishmania*-infected macrophages. BMDMs were then infected with stationary phase promastigotes *L.* (*L.*) *amazonensis PH8*, *2269* strains and *L.* (*L.*) *infantum* at a 10:1 an parasite: macrophage ratio and incubated for 24 h at 34 °C and 5% CO_2. The cells were washed with PBS 1× to remove non-interiorized promastigotes and fresh medium was added, followed by incubation with p-Acl or p-Acl R7 (0, 2.5, 50, 100, 150 μM) again in the same conditions for another 24 h. Coverslips containing infected cells were then washed with PBS 1×, fixed and stained (Giemsa; Merck). The number of amastigotes was determined by counting 300 macrophages in triplicates using Leica LAS Core microscope system. Infection index was obtained by multiplying the number of infected cells by the number of amastigotes per 100 host cells and expressed as percentages of untreated control cells (100%).

2.9. Determination cytotoxicity (CC_{50}) against BMDMs and selectivity index

BMDMs were seeded into 96-well culture plates (5×10^5 cells/well) for 3 h in a 5% CO_2 atmosphere at 37 °C. The BMDMs were incubated with increasing concentrations of peptides for 24 h under the same incubation conditions detailed above. Untreated cells were included as negative controls. The MTT assay was performed and CC_{50} values were calculated using a nonlinear regression curve and the selectivity index (SI) values were determined according to the formula = CC_{50}/EC_{50}.

2.10. Assessment of plasma membrane permeabilization by peptides

Plasma membrane permeabilization of mammalian cells and parasites to ethidium bromide (EB) was investigated in a Hitachi F-2500 fluorescence spectrophotometer at 37 °C: (excitation = 590 nm, emission = 560 nm) as described by Cohen et al. (1990) with some modifications. Promastigotes of *L.* (*L.*) *amazonensis* (*PH8*) and BMDMs were centrifuged at 1500 ×*g* for 10 min and washed with buffer solution pH 7.6 prepared with 11 mM KCl,140 mM NaCl and Tris-HCI at 75 mM. Using the same solution with $C_6H_{12}O_6$ (10 mM), parasites were re-suspended into a cuvette (10^7 cells/mL). Each peptide at 50 μM was added to the plates with 10 μM EB. An increase in fluorescence intensity means higher DNA accessibility, which was quantified and monitored as a function of time and expressed as fluorescence units (FU). The maximal permeabilization and nucleic acid binding was measured after the addition of 100 mM digitonin.

2.11. Statistical analysis

Statistical analysis, when applicable, were done by one-way analysis of variance (ANOVA) with Tukey's Honest Significant Difference test (posttest) using GraphPad Prism software. Differences with $P < 0.05$ were considered significant.

3. Results

3.1. Synthesis and characterization of 13-mer peptides

Peptide p-Acl (KKYKAYFKFKCKK-NH_2) and its analogue p-AclR7 (**RRY**RAYF**R**F**R**C**RR**-NH_2) were synthesized as *C*-terminal carboxamides by standard solid-phase peptide synthesis (SPPS) approaches using the Fmoc/tBu orthogonal protection scheme (Almeida et al., 2018a). After purification, both peptides were obtained in high purity (*ca.* > 97.5%), moderate yields (*ca.* > 30%) (Fig. S1 and Table S1), and presenting mass spectrometric data in agreement with their structures (Fig. S2).

In silico prediction of the physicochemical properties for these peptides using PepDraw Program indicate that, despite both 13-mers have the same net charge (+8), their isoelectric points and hydrophobicity values (Wimley-White scale) differ, in consequence of the Lys → Arg replacements made. As such, p-AclR7, where 7 out of 13 amino acid residues are arginines, has higher isoelectric point (pI = 12.61) and lower hydrophobicity (+ 16.21 kcal*mol^{-1}) than p-Acl, where Arg residues are absent and 7 out of 13 amino acids are lysines (Table 1).

Relevantly, p-Acl and p-Acl R7 share structural characteristics with peptide-based antileishmanial agents reported in the literature (Luque-Ortega et al., 2003; Mangoni et al., 2005; Costa et al., 2008; Perez-Cordero et al., 2011; Marr et al., 2016). Many of those peptides are short and composed by positively charged and hydrophobic residues, respectively identified by pink and red colors in the multiple sequence alignment shown in Fig. 1.

3.2. Effect of 13-mer synthetic peptides on promastigotes

The leishmanicidal potential of the 13-mer cationic peptides was evaluated against promastigotes of (i) two strains (PH8 and 2269) of *L.* (*L.*) *amazonensis*, the etiological agent of localized and diffuse

Table 1
Peptide physicochemical parameters as predicted by PepDraw Program.

Peptides	Charge	pI	Hydrophobicity (kcal*mol^{-1})
p-Acl	+8	10.76	+23.14
p-AclR7	+8	12.61	+16.21

Peptides	Primary structure	References
CM2	-KWKLFKKVLKVL---	(Luque-Ortega et al., 2003)
Cm3	-KWKLFKKILKVL---	(Luque-Ortega et al., 2003)
CM1	-KWKLLKKIGAVLKVL	(Luque-Ortega et al., 2003)
Temporin A	-FLPLIGRVLSGIL--	(Mangoni et al., 2005)
p-MTXI	RKYMAYLRVLCKK---	(Costa et al., 2008)
p-Acl	KKYKAYFKFKCKK---	(Present work)
p-MTXII	KKYRYHLKPLCKK---	(Costa et al., 2008)
p-AclR7	RRYRAYFRFRCRR---	(Present work)
Temporin B	-LLPIVGNLLKSLL--	(Mangoni et al., 2005)
E6	RRWRIVVIRVRR----	(Marr et al., 2016)
Pr-3	VSRRRRRRGGRRRR--	(Perez-Cordero et al., 2011)
RI-1018	RRWIRVAVILRV----	(Marr et al., 2016)

Fig. 1. Multiple sequence alignment of synthetic peptides addressed in this study with 12-15mer leishmanicidal peptides reported in the literature. Alignment performed by using the CLUSTAL 2.1 multiple sequence alignment tool.

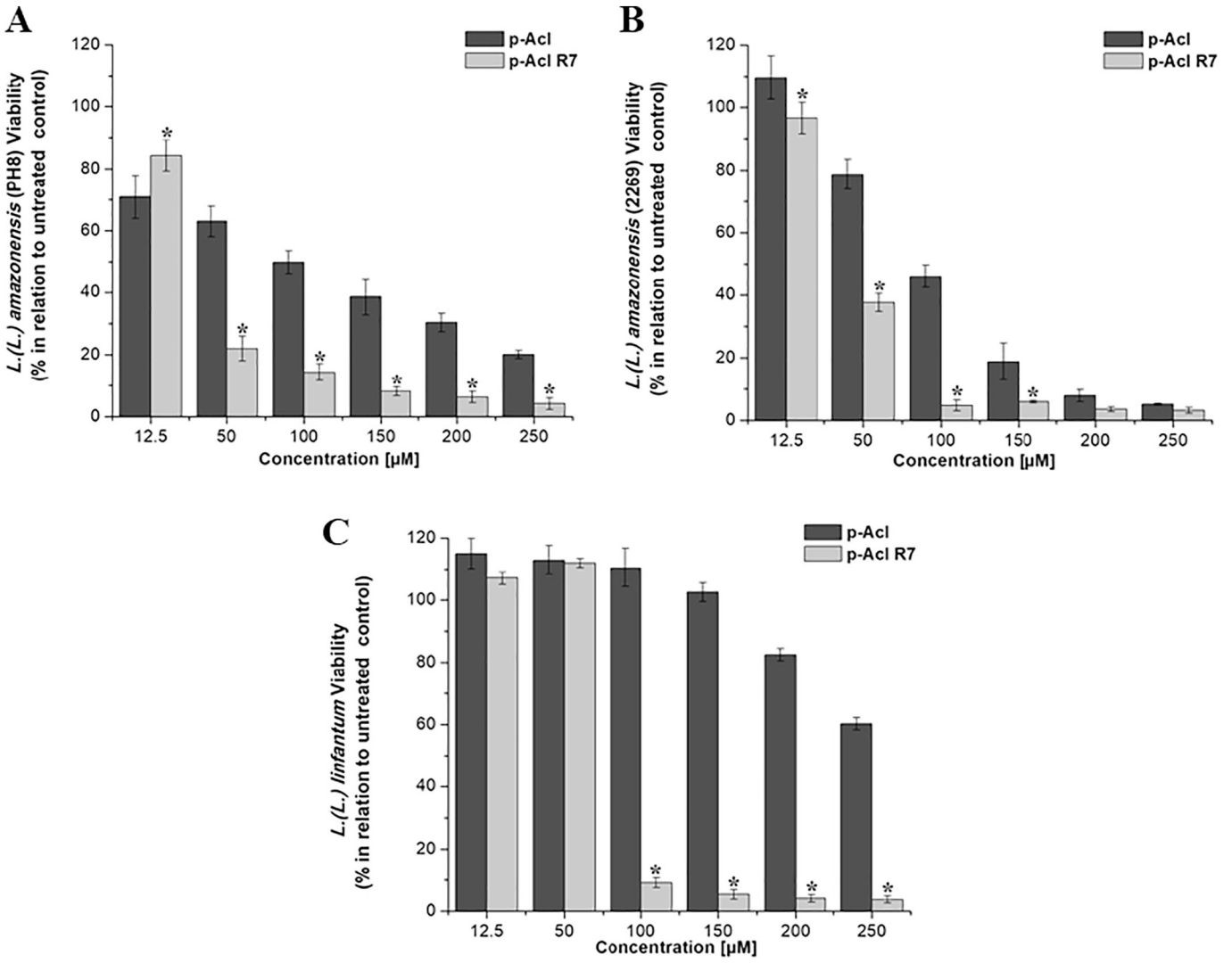

Fig. 2. Antileishmanial activity of p-Acl and p-AclR7 against *Leishmania* promastigotes. Early-logarithmic-phase promastigotes of *L.* (*L.*) *amazonensis PH8* (A), *2269* (B) and *L.* (*L.*) *infantum chagasi* (C) were exposed to the 13-mer peptides at different concentrations (0 to 250 μM) for 24 h. Control was considered as 100% of cell viability (untreated). Viability was determined by the MTT colorimetric assay and the EC_{50} values of each assay were calculated using GraphPad Prism. Bars represent the mean viability ± SD. Experiments were performed at least twice in triplicates. Asterisks indicate a statistically significant ($p < 0.05$) difference in values, in comparison to the p-Acl group.

cutaneous leishmaniasis in the Americas; and (ii) *L.* (*L.*) *infantum chagasi*, the causative agent of visceral leishmaniasis in the Americas. Fig. 2 shows that p-Acl and p-AclR7 induced a dose-dependent reduction in the number of viable cells of all *Leishmania* species tested after 24 h incubation. pAcl showed EC_{50} values of 50.98, 57.23 and 220.32 μM against *L.* (*L.*) *amazonensis 2269* and *PH8*, and *L.* (*L.*) *infantum chagasi*,

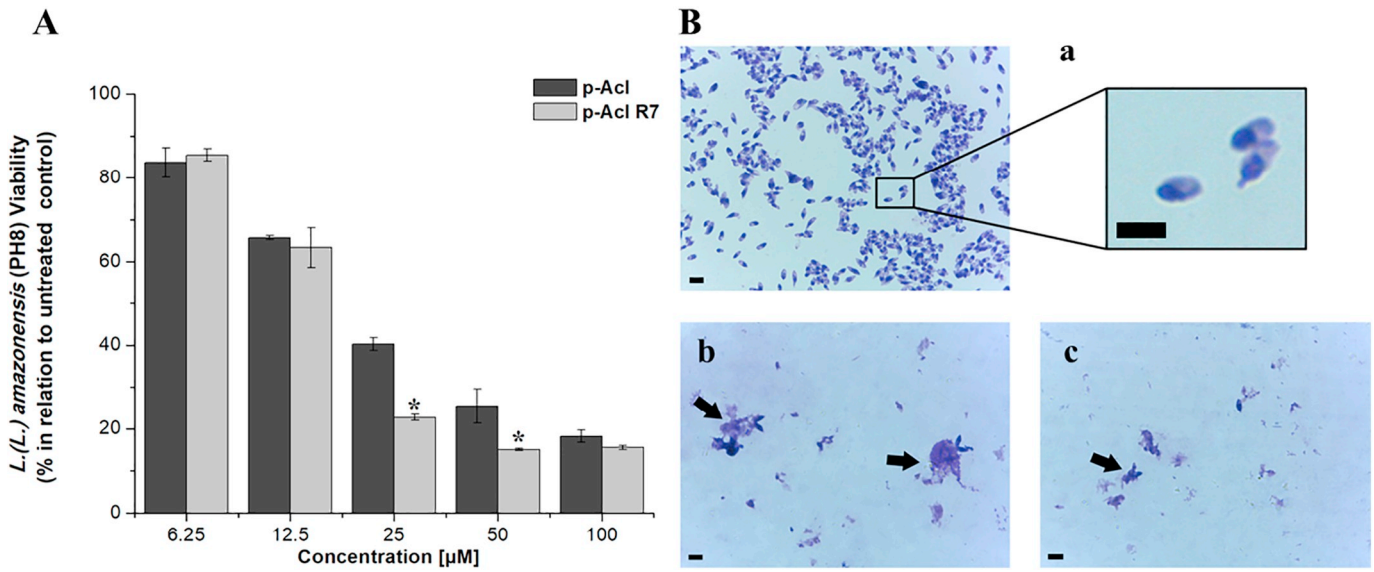

Fig. 3. Antileishmanial activity of p-AcI and p-AcIR7 against *L.* (*L.*) *amazonensis PH8* amastigotes recovered from skin lesions. (A) *L.* (*L.*) *amazonensis PH8* amastigotes were incubated in 96-well plates with the synthetic short peptides (0–100 μM) for 24 h at 32 °C. Control was considered as 100% of cell viability (untreated). Amastigotes viability was determined by the MTT colorimetric assay and the EC_{50} values calculated for each assay using GraphPad Prism. Bars represent the mean viability ± SD. Experiments were performed at least twice in triplicates. (B) Photomicrographs obtained using a Leica LAS Core microscope system show untreated *L.* (*L.*) *amazonensis PH8* amastigotes (a) and parasite debris after incubation with p-AcI (b) and p-AcIR7 (c). Bar = 4 μm. Asterisks indicate a statistically significant ($p < 0.05$) difference in values, in comparison to the p-AcI group.

respectively. Interestingly, its analogue p-AcIR7 showed higher activity, by presenting lower EC_{50} values = 27.19, 36.83 and 70.71 μM for *L.* (*L.*) *amazonensis PH8* and *2269*, and *L.* (*L.*) *infantum chagasi*, respectively. Routinely, promastigotes incubated with Amphotericin B for 24 h are included as positive control groups, showing EC_{50} values that ranged from 0.27 to 0.53 μM for both species and strains here used.

3.3. Effect of 13-mer synthetic peptides on amastigotes recovered from skin lesions

The leishmanicidal effect of the two synthetic peptides was also investigated in lesion-recovered *L.* (*L.*) *amazonensis PH8* amastigotes. The cationic peptides triggered a significant reduction in cell viability in a dose-dependent manner, as determined by the MTT method (Fig. 3A). p-AcIR7 was slightly more active than the native sequence p-AcI at 25 and 50 μM, in accordance with respective *in vitro* EC_{50} values, 15.62 and 17.69 μM. Reduction of cell viability after treatment with the peptides can also be visualized in the photomicrographs in Fig. 3B, for which culture smears were fixed and stained for parasite morphology examination.

3.4. Effect of 13-mer synthetic peptides to host cells and intracellular amastigotes

In vitro assays were performed aiming at evaluating the cytotoxicity of the 13-mer peptides to murine primary macrophages (host cells to *Leishmania* spp. amastigotes). Briefly, Balb/C mice bone marrow-derived macrophages (BMDMs) were incubated with increasing concentrations of each 13-mer peptide for 24 h at 37 °C. Significant reduction in metabolic activity of the BMDMs cells were visualized at the concentration of 200 μM (data not shown). The host cells were slightly more susceptible to p-AcI (CC_{50} = 232.88 μM), than to p-AcIR7 (CC_{50} = 273.70 μM). Incubation of BMDMs with up to 150 μM of the 13-mers did not show any significant toxic effects. Based on these findings, peptides were tested for their ability to inhibit intracellular parasites at 50, 100 and 150 μM. In this case, infection index was calculated upon counting the number of infected cells and intracellular parasites for untreated and peptide-incubated infections.

Both peptides were found to reduce the intracellular parasite burden, with peptide p-AcIR7 displaying a more pronounced killing effect, specially at 50 μM for *L.* (*L.*) *amazonensis*, as compared to the control untreated group (Fig. 4). *L.* (*L.*) *infantum* intracellular amastigotes were less sensitive to p-AcI and p-AcIR7 when compared to *L.* (*L.*) *amazonensis*. Nevertheless, the native p-AcI sequence also significantly reduced macrophage infection indices, as compared to controls ($p < 0.05$). Fig. 4D shows microscopic visualization of parasite burden reduction on macrophages incubated with the peptides.

Based on these experimental results with infected-BMDMs and CC_{50} values determined in amastigote and promastigote assays, selectivity index (SI) were calculated and presented in Table 2.

3.5. Plasma membrane permeabilization by 13-mer peptides

In order to obtain insights into molecular mechanism of action of 13-mer peptides, the incorporation of ethidium bromide (EB) was monitored by a spectrofluorometric assay after exposure of promastigotes and BMDMs to 13-mer peptides and EB. Both short peptides affected only of parasite plasma membrane integrity as can be seen in Fig. 5. Fluorescence emission signals were increased after incubating the parasites with 50 μM of each peptide. p-AcIR7 incubation led to a faster and more abundant EB incorporation when compared with p-AcI. Fluorescence signal was not observed when BMDMs were incubated with EB and peptides (50 μM; data not shown).

4. Discussion

Leishmaniasis remains as a major socioeconomic and life-threatening burden in many countries, for which treatment options are limited and far from ideal (Okwor and Uzonna, 2016). Despite many collective efforts, current therapies are characterized by toxicity, high cost, parasite resistance, and undesirable side effects and treatment lengths. Therefore, design and discovery of safer and more effective chemotherapeutic agents to address this relevant obstacle endures as a global health challenge and an urgent need for researchers and pharmaceutical companies (Siqueira-Neto et al., 2010). In this sense, biomolecules from natural products, such as plants (Graebin et al., 2010;

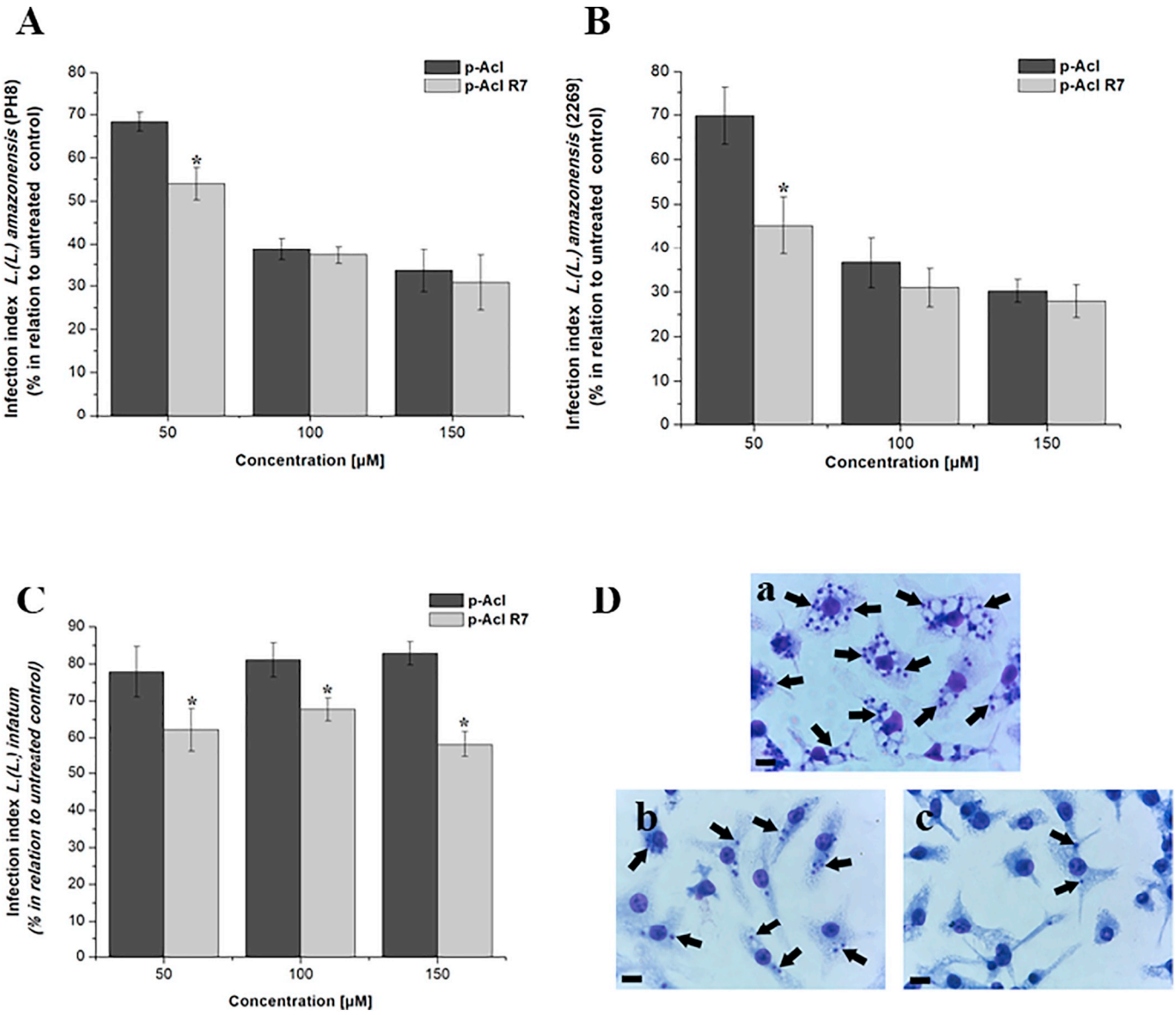

Fig. 4. Activity of the 13-mer peptides against intracellular *Leishmania* spp. amastigotes. BMDMs were infected with *L.* (*L.*) *amazonensis PH8* (A), *2269* (B) and *Leishmania* (*L.*) *infantum* (C) promastigotes, and incubated with different concentrations of 13-mer peptides for 24 h to evaluate effects on progression into the amastigote stage. Cells were washed, fixed, stained and the infection index determined by microscopic analysis. Bars represent the mean viability ± SD. Experiments were performed at least twice in triplicates. (D) Representative photomicrographs obtained using a Leica LAS Core microscope system show untreated infected BMDMs (a) and decrease of infected cells/amastigotes when treated with p-AcI (b) and p-AcIR7 (c). Black arrows point to intracellular amastigotes. Bar = 10 μm. Asterisks indicate a statistically significant ($p < 0.05$) difference in values, in comparison to the p-AcI group.

Table 2
Selectivity indices of synthetic peptides on extra- and intracellular forms of *Leishmania* spp.

Selectivity index	p-AcI	p-AcIR7	*Leishmania* sp.
SI^1	1.1	4.7	*L.* (*L.*) *infantum*
SI^1	4.5	8.0	*L.* (*L.*) *amazonensis 2269*
SI^1	4.1	10.8	*L.* (*L.*) *amazonensis PH8 L.* (*L.*) *amazonensis*
SI^2	13.2	18.9	*PH8*

(SI^1) = CC_{50} BMDMs/EC_{50} for *Leishmania* spp. promastigotes.
(SI^2) = CC_{50} BMDMs/EC_{50} for *L.* (*L.*) *amazonensis PH8* amastigotes.

Gontijo et al., 2012), marine animals (Tempone et al., 2011), amphibians (Calhoun et al., 2016) and reptiles (Adade et al., 2014) has been receiving great attention as scaffolds for the investigation of leishmanicidal agents.

Snake venom phospholipases (PLA_2s) are biomolecules recognized by their functional versatility and applications (Almeida et al., 2018b), ranging from their relationship to snakebite accidents (Almeida et al., 2016b) to biomedical and biotechnological investigations (Corrêa et al., 2016). PLA_2s Ly49 are toxins without catalytic properties but with a broad repertoire of biological activities, including leishmanicidal activity (Lomonte et al., 2009). Their mechanism of antileishmanial action is not fully elucidated and remains a matter of study. Membrane perturbations caused by specific and small regions of PLA_2s has been the main mechanism of action proposed for these proteins (Lomonte et al., 2010), which in turn has been linked to the excellent antileishmanial activity found for short peptides mimicking the C-terminal region of PLA_2s (Costa et al., 2008).

Considering that peptide therapy has been explored towards the development of leishmanicidal agents (Marr et al., 2012; Ruiz-Santaquiteria et al., 2018), we decided to evaluate synthetic 13-mer

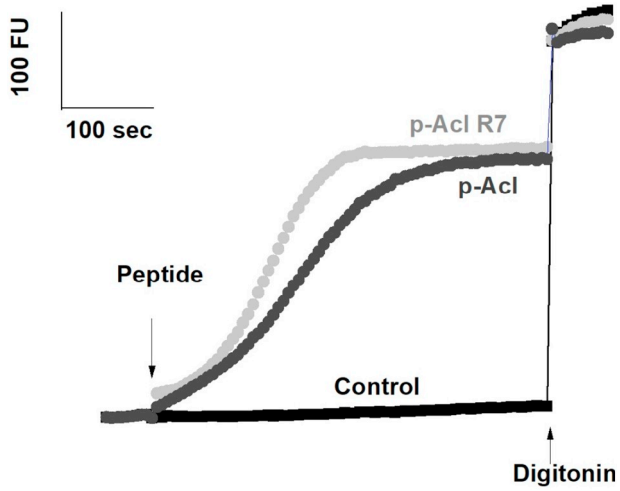

Fig. 5. Plasma membrane permeabilization of *L.* (*L.*) *amazonensis PH8* promastigotes upon exposure to 13-mer peptides. Peptides destabilized parasite cell membranes and allowed incorporation of EB. Control (black line), p-AclR7 50 μM (light gray line) induced faster and greater damage than p-Acl 50 μM (dark gray line). Digitonin (1.6 mM) was added indicated by the arrow to allow maximal permeabilization.

amide peptides, inspired in the C-terminal region of the ACL protein as antileishmanial leads. ACL retains structural and functional characteristics of the PLA_2 superfamily, for which the ability to interact with phospholipids and damage cell membranes has been associated to electrostatic interactions with C-terminal lysines (Ambrosio et al., 2005). Previous studies have reported that this molecular region of ACL, comprising residues 115–129, mediates Ca^{2+} influx and reduces C2C12 myotubes viability by 40% at 250 μg/ml of peptide (Cintra-Francischinelli et al., 2010). The C-terminal region of ACL, as of other PLA_2 toxins from snake venoms, share structural and biochemical features with amphipathic cationic AMP, many of which have antiprotozoal action (Torrent et al., 2012). Our results confirm this trend, as we demonstrate the leishmanicidal potential of p-Acl. Its sequence matches that of the 115–129 segment in ACL, and of its analogue p-AclR7, where all native lysines have been replaced by arginines, hence possessing a similar net charge but higher isoelectric point and lower hydrophobicity.

Both peptides not only presented good activity against promastigotes of *Leishmania* but also reduced macrophagic infection levels. EC_{50} values were in accordance with the range found for other synthetic antileishmanial peptides, such as dermaseptin, Pr-2, Pr-3, E6, L-1018 and LL-37 (Perez-Cordero et al., 2011). Concentration-dependent reduction of promastigotes and amastigotes' viability was also similar to that of other peptides, such as BMAP-28; however, it is relevant to consider that a limited concentration range of BMAP-28 isomers has been tested against an Old World *Leishmania* species (*L. major*), showing up to 82% of intracellular parasite burden reduction (Lynn et al., 2011).

In general, studies investigating leishmanicidal properties of peptides derived from C-terminal region of PLA_2s focus primarily on *Leishmania* promastigotes (Costa et al., 2008). To the best of our knowledge, this is the first time that peptides derived from PLA_2 toxins are further tested against intracellular amastigotes and in macrophage infection assays for two very distinct *Leishmania* species. *Leishmania* amastigotes survive within macrophages and to assess activity against this parasite stage is mandatory when finding new candidates for leishmanisasis chemotherapy. On the other hand, it is important to ensure that any compound active against *Leishmania* is safe enough for the mammalian host cells. As such, 13-mer peptides were further tested for their toxicity to macrophages, exhibiting good selectivity indices. Such selectivity may result from differences in membrane lipid compositions of parasite *versus* mammalian cells investigated, in line with reports where therapeutics effects induced by many cationic peptides have been associated with their affinity to variable lipid contents of cell membranes (Oliveira et al., 2016). For instance, a recent study using high resolution microscopy has demonstrated the preferential affinity of ocellatin-PT peptides from frog skin secretions to *Leishmania* membranes compared to the mammalian model (Oliveira et al., 2016).

Membrane selectivity towards parasite's components is considered a very interesting target. Martins and colleagues have hypothesized that aminophospholipids, abundant in parasite membranes, may interact with DECP-11 leading to critical disturbs in mimetic parasite membranes (Martins et al., 2017). Broad lipidomics studies have revealed that *Leishmania* membranes have a lipidic profile distinct from those of mammalian cells, showing predominance of phosphatidylcholine and phosphatidylethanolamine (Zheng et al., 2010; Imbert et al., 2012). In addition, mammalian cells incorporate cholesterol in their lipidic compositions, while *Leishmania* and other trypanosomatids use ergosterol instead (McCall et al., 2015). Currently, full lipid profiling of different cell membranes is being undertaken by means of several scientific tools, in order to obtain clues for the optimization and development of AMP (Besenicar et al., 2006; Suwalsky et al., 2015; Oliveira et al., 2016).

Cationic peptides have been reported to owe their leishmanicidal effects to different modes of action, involving or not binding cellular targets, activation of apoptosis signaling pathways, membrane disruption, synergism events or other indirect effects (Marr et al., 2012). To investigate if the 13-mer peptides owed their antileishmanial properties to cell membrane permeabilization, spectrofluorometric assays were undertaken. We demonstrated that both peptides permeabilize parasites' membranes, as reported for several leishmanicidal AMP (Lynn et al., 2011; Marr et al., 2012). For instance, leishmanicidal activity of temporins, a promising family of AMP, has been linked to a membranolytic mechanism (Copolovici et al., 2014). At the same concentration (50 μM), our peptides did not permeabilize the host cell membrane, which agrees with our cytotoxicity data.

Although both peptides presented interesting antileishmanial activity and low cytotoxicity, p-Acl displayed slightly less activity and selectivity as compared to its analogue, p-AclR7. Both are composed by hydrophobic and positively charged similarly to other reported leishmanicidal peptides, like CM2 (Luque-Ortega et al., 2003), p-MTXI (Costa et al., 2008), p-MTXII (Costa et al., 2008), or temporin B (Mangoni et al., 2005), among others. The cationicity and amphipathicity are key features shared between these peptides, and are hallmarks of both p-Acl and p-AclR7. Still, arginine-rich peptides have stood out and revealed important clues in various lines of research and for drug development (Ruiz-Santaquiteria et al., 2018) mainly owing to their intrinsic ability of establishing strong interactions with cell membranes (Copolovici et al., 2014). Previous works have shown that despite the physicochemical similarity between arginine and lysine, these amino acid residues can provide variable potency, distinct functional properties to the peptide and can affect the ability of interacting and disrupting the membrane (Deslouches et al., 2016; Ojeda et al., 2017). Studies conducted by Su et al. (2009), Mitchell et al. (2000) and Kalia et al. (2003) have shown that arginine-rich sequences have better membrane-disrupting properties than lysine-rich peptides, which comes into agreement with our findings for p-Acl and p-AclR7. These are identical in terms of length and overall charge, however they exhibit different pI and hydrophobicity values. The favorable effects of arginine residues in membrane-active peptides, like AMP and CPP, have been mainly attributed to their side-chain guanidine group, which favors proper interaction with surface-exposed elements of cell membranes, such as lipids and proteins (Ojeda et al., 2017). Functional studies have demonstrated that polyarginine peptides of general formula $(Arg)_n$ enter cells more efficiently than their respective polylysine analogs of the same length (Mitchell et al., 2000). In this sense, it is reasonable to assume that the enhanced antileishmanial activity and selectivity of p-AclR7 over p-Acl is due to an improved ability of the former to interact

with parasite's membrane with higher affinity and specificity than the latter. Further studies may be conducted to determine the potential aggregation of 13-mer peptides to parasite mimetic-membranes in order to advance in the comprehension of our peptides leishmanicidal effect.

5. Concluding remarks

Taken together, our results confirm the potential of 13-mer cationic peptides inspired in the C-terminal region of reptilian PLA_2 toxins as leishmanicidal agents. It further highlighted the role that replacement of lysine by arginine residues may assume in the optimization of short cationic peptides, which may be a promising strategy in the development and rational design of therapeutic molecules for neglected diseases.

Acknowledgments

The authors thank to the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq; Process #202831/2014-0) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for funding part of this work and fellowships. NV acknowledges support from Fundação para a Ciência e a Tecnologia (FCT, Portugal) and FEDER (European Union) in the framework of the projects "Institute for Research and Innovation in Health Sciences" (POCI-01-0145-FEDER-007274) and IF/00092/2014/CP1255/CT0004. PG thanks FCT for financial support to the LAQV-REQUIMTE Research Unit (UID/QUI/50006/2013). DCM is a recipient of a Young Investigator Award from Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP; Process #2014/21129-4).

Declaration of competing interest

The authors declare no conflict of interest to this study.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cbpc.2019.108612.

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