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BIOACTIVE PEPTIDES INSPIRED ON A LYS49 PHOSPHOLIPASE A_2 AGAINST BACTERIA AND CANCER CELLS

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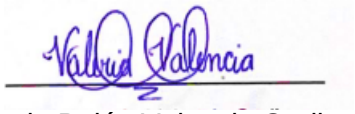

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RESUMEN

Los casos de incidencia y mortalidad causados por las infecciones bacterianas y el cáncer representan desafíos a la salud que amenazan el desarrollo socioeconómico global. Por ello, la industria farmacéutica se ha enfocado en la caracterización de moléculas bioactivas de origen natural para el descubrimiento de terapias emergentes. Las fosfolipasas A_2 (PLA_2) son enzimas presentes en el veneno de serpiente que han demostrado ser útiles para el diseño de fragmentos cortos potencialmente terapéuticos. En esta investigación, se sintetizaron dos fragmentos análogos de la región C-terminal de una PLA_2 Lys49 aislada en el veneno de *Crotalus oreganus abyssus*, denominados pC-CoaTxIIR2 y pC-CoaTxIIR6. Se utilizaron predictores bioinformáticos y ensayos *in vitro* para determinar las actividades: hemolítica, antimicrobiana y anticancerígena de los péptidos sintéticos. Los rangos seleccionados para los experimentos de inhibición bacteriana y tumoral no demostraron una disrupción eritrocitaria significativa. Posteriormente, el potencial bactericida de los péptidos se evaluó en cepas de *Staphylococcus aureus* y *Escherichia coli*. Los resultados indicaron que pC-CoaTxIIR6 mostró mayor inhibición para el crecimiento de las bacterias: *S. aureus* (MIC = 50 µM) y *E. coli* (MIC = 75 µM). Determinando que, las sustituciones de los residuos de lisina por aminoácidos de arginina contribuyen al potencial antibacteriano. En cambio, los ensayos citotóxicos demostraron que la conservación de residuos de lisina en la cadena de los péptidos derivados de la C-terminal de la PLA_2 Lys49 reducen la actividad metabólica de la línea tumoral (SH-SY5Y). Nuestros hallazgos señalan que, pC-CoaTxIIR2 (EC_{50} = 371,2 µM) posee mayor función citotóxica que pC-CoaTxIIR6 (EC_{50} = 741,9 µM). Por lo tanto, los péptidos inspirados de Lys49 PLA_2 pueden ejercer efectos multifacéticos prometedores. Por esta razón, son de interés para la exploración y el desarrollo de nuevas estrategias terapéuticas para tratar enfermedades emergentes.

Palabras clave: Lys49-PLA_2, C-terminal, péptidos, antibacteriano, anticancerígeno.

ABSTRACT

The incidence and mortality caused by bacterial infections and cancer represent health challenges that threaten global socioeconomic development. Therefore, the pharmaceutical industry has focused on the characterization of bioactive molecules of natural origin for the discovery of emerging therapies. Phospholipases A_2 (PLA_2) are enzymes present in snake venom that have proven to be useful for the design of potentially therapeutic peptides. In this research, two fragment analogs of the C-terminal region of a Lys49-PLA_2 isolated from the venom of *Crotalus oreganus abyssus*, named pC-CoaTxIIR2 and pC-CoaTxIIR6, were synthesized. Bioinformatic predictors and *in vitro* assays were used to determine the hemolytic, antimicrobial and anticancer activities of the synthetic peptides. The ranges selected for bacterial and tumor inhibition experiments did not demonstrate significant erythrocyte disruption. Subsequently, the bactericidal potential of the peptides was evaluated in *Staphylococcus aureus* and *Escherichia coli* strains. The results indicated that pC-CoaTxIIR6 showed higher inhibition for the growth of the following bacteria: *S. aureus* (MIC = 50 µM) and *E. coli* (MIC = 75 µM). Considering that, substitutions of lysine residues by arginine amino acids contribute to the antibacterial potential. In contrast, cytotoxic assays demonstrated that the conservation of lysine residues in the chain of peptides derived from the C-terminal Lys49-PLA_2 reduced the metabolic activity of the tumor line (SH-SY5Y). Our findings indicate that, pC-CoaTxIIR2 (EC_{50} = 371.2 µM) possesses greater cytotoxic function than pC-CoaTxIIR6 (EC_{50} = 741.9 µM). Therefore, Lys49-PLA_2 inspired peptides may exert promising multifaceted effects. For this reason, they are of interest for the exploration and development of new therapeutic strategies to treat emerging diseases.

Keywords: Lys49 PLA_2s, C-terminal, peptides, antibacterial, anticancer.

INTRODUCTION

Despite the successes in the field of medicine, the incidence and mortality caused by bacterial infections and cancer represent threats to global health and socioeconomic development [1,2]. According to estimates of recent studies, these diseases are going to be the main causes of death in the coming decades, due to adverse reactions and resistance produced by currently available treatments [3–5]. Therefore, the research for new alternatives that provide immediate solutions to these challenges is required [6–8]. In this context, snake venom constitutes a relevant source of bioactive molecules that contribute to the development of specific prototypes for innovative clinical approaches [9–11].

Snake venoms are composed of a cocktail of functional molecules such as phospholipases A_2 (PLA_2s), serine proteases (SVSPs), metalloproteinases (SVMPs) and three-finger peptides (3FTX), which are responsible for neurotoxic, myotoxic, cardiotoxic and hematotoxic activities [10–12]. Interestingly, several of these isolated compounds have shown functional pharmacological properties [12,13]. In particular the PLA_2s family, which are usually the most abundant component in snake venom and responsible for inducing changes in the composition of cell membranes [14–16]. According to the hydrolytic effect and calcium binding site, this toxin is divided into two isoforms: Asparagine-49 phospholipase A_2 (Asp49 PLA_2) catalytically active, and Lysine-49 phospholipase A_2 (Lys49 PLA_2) catalytically inactive [12,17]. Despite their different enzymatic activities, Asp49 PLA_2 and Lys49 PLA_2 have demonstrated lytic activities for different types of pathogens [12]. Therefore, phospholipase A_2 isoforms function as attractive templates for obtaining bioactive peptides with therapeutic potential [10,17,18].

Pharmacological studies based on design of peptides inspired by the Lys 49 PLA_2 protein, aim at mimicking the protein region located at the C-terminus, which is responsible for its toxic effects [19–21]. For instance, the pC-CoaTxII peptide inspired by the CoaTxII toxin from the snake *Crotalus oreganus abyssus*, to efficiently inhibit the growth of multidrug-resistant clinical isolates [13,20]. Another example is the peptide analog p-AclR7, which showed enhanced leishmanicidal activity on promastigotes of *L. (L.) amazonensis PH8* and *2269*, and *L. (L.) infantum chagasi*, after substitution of lysine residues by arginine [18].

In this context, the activity of biomimetic peptides is mainly characterized by positive charge and the presence of hydrophobic residues [18,19]. These physicochemical properties are similar in Lys49 PLA_2-inspired short fragments and antimicrobial peptides (AMPs). Therefore, AMPs have been considered for exploration as promising dual antitumor therapies, as demonstrated by peptides: p-AppK and pEM-2_D [15,17,19]. Consequent to this recognition, these synthetic molecules are considered as prokaryotic cell membrane permeabilizing agents [22,23].

Considering this background, the hemolytic, antibacterial and anticancer activities of two Lys49 PLA_2-inspired peptides were analyzed, synthesized and evaluated using online bioinformatics tools and *in vitro* assays. The first analogue peptide pC-CoaTxIIR2 corresponding to modifications in the sequence of the C-terminal region of CoaTxII toxin, by changing arginine residues to lysine residues; and the peptide pC-CoaTxIIR6, where all native lysine amino acids were replaced by arginine. The antibacterial potential was determined against Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Escherichia coli*) bacteria, and the cytotoxic activity was evaluated in neuroblastoma cell line (SH-SY5Y).

METHODS

Peptide design and synthesis

Two peptides inspired on the primary structure of CoaTx-II protein were selected for the peptide synthesis and evaluation of antibacterial and cytotoxic effects. Analogues were synthesized following the protocol previously used in our research group [17,20]. Automated peptide synthesis was performed according to the solid-phase peptide synthesis (SPPS) method, using Fmoc technology on Rink Amid Novabiochem resin (0.60 mmol/g) with the Liberty Blue microwave peptide synthesizer (CEM Corporation). Fmoc protector groups were eliminated with piperidine. Subsequently, the peptides were cleaved from resin using 95 % trifluoroacetic acid (TFA), 2.5 % water and 2.5 % triisopropylsilane (TIS). Finally, the crude peptides were precipitated with cold diethyl ether and freeze-dried for 24 h at -80 °C at 0.09 mT pressure.

Bioinformatic analysis

Virtual bioinformatic tools were used to perform primary structure and properties analysis of the two venom toxin-derived peptides, pC-CoaTxIIR2 and pC-CoaTxIIR6. PepCalc (https://pepcalc.com) and PepDraw (http://www.tulane.edu/~bochem/WW/PepDraw) were run to determinate the physicochemical properties (peptide mass, charge, isoelectric point and hydrophobicity). The functional study of antimicrobial activity was predicted with AMP Scanner (www.ampscanner.com) and AMPfun (http://fdblab.csie.ncu.edu.tw/AMPfun/run.html). AntiCp (https://webs.iiitd.edu.in/raghava/anticp/index.html) and AcPred (http://codes.bio/acpred) were used to verify the anticancer effect. The alignment of analogues with other functional peptides against bacteria and cancer cells were analyzed by Clustal omega. The hemolytic activity was simulated using HemoPI (https://webs.iiitd.edu.in/raghava/hemopi/), HemoPred (http://codes.bio/hemopred/) and HLPpred-Fuse (http://thegleelab.org/HLPpred-Fuse/). Additionally, PEP2D (https://webs.iiitd.edu.in/raghava/pep2d/) and I-Tasser (https://zhanggroup.org/I-TASSER/) were used to recognize the structure of the peptides. Finally, the cell penetration capacity of the sequences was evaluated in the computational tool BChemRF-CPPred (http://comptools.linc.ufpa.br/BChemRF-CPPred/).

Peptides purification

The purity of the synthetic products was analyzed by reverse phase high pressure liquid chromatography (RP-HPLC) with a C18 analytical column (250 x 4.6 mm). Approximately, 0.5 mg of each crude peptide was dissolved in acetic acid solution (1 %) and 50 µL was injected into the system. Analogues were eluted with a linear gradient formed from 95 % mobile phase A (99.9 % water, 0.1 % formic acid) to 100 % mobile phase B (99.9 % acetonitrile, 0.1 % formic acid), at a flow rate of 1 mL/min for a period of 60 minutes and fractions were collected each minute. During the run, the fractionation was monitored at 220 nm (UV-VIS detector, 1260 Infinity II, Agilent). The purity process was repeated using FLASH equipment (BUCHI), if necessary, until obtaining a purity higher than 90 %. This system was fitted with a C18 column (7 × 2 cm, 40 µm, 4 g). After the coupling, 10 mg of crude peptides were diluted in 1 mL of acetic acid (1 %) and filtered using 0.4 µm membrane. The same buffers and linear gradient were employed at a flow rate of 2.5 mL/min for 30 minutes. The peptides were lyophilized at -80°C and 0.09 mbar [17].

Determination of intact mass

Identification of the intact mass of the purified peptides was determined by Matrix-assisted laser ionization/desorption time of flight mass spectrometry MALDI-TOF MS (Axima Confidence, Shimadzu). Two microliters of each peptide mixed with 1 µL matrix of a-cyano-4-hydroxycinnamic acid (CHCA, 10 mg/mL) was placed into the plate. The MALDI target plate was monitored at 500 – 3500 Da per well with duplicate verification, 40 Watts of voltage.

Hemolytic assay

Hemolytic activity protocol was performed as previously reported by Proaño [24]. The membrane lysis of erythrocytes was determined using 200 µL of a suspension of 2 % human red blood cells (type O+) diluted in 200 µL of each peptide concentration (1, 3, 6, 12, 25, 50, 75, 150, 250, 300 µM) and incubated at 37°C for 2 hours. After the incubation, the cells were centrifuged at 1000 rpm for 5 minutes and the supernatant was transfered to a 96-well microplate. The hemolytic activity was measured at 550 nm using a GloMax Multi Detection System (Promega). PBS and (2 % v/v) Triton X-100 were used as negative and positive control, respectively. Each peptides concentration and controls were performed in triplicate.

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) assays

The antimicrobial activity of peptides was evaluated against *Staphylococcus aureus ATCC 25923 and Escherichia coli ATCC 25922*. The microorganisms were cultured following the protocol previously used in our group [24]. Bacteria were cultured until reach the density of 1 x10^6 CFU/mL and the peptides were dissolved in dimethyl sulfoxide (DMSO) at the following concentrations: 1.625 – 300 µM. Therefore, 198 µL of each microorganism were diluted with 2 µL of peptides and incubated for 18 h at 37°C in a 96-well sterile microplate. The positive control was sterile Mueller Hinton Broth (MHB) and the negative control was bacterial culture with DMSO. Before reading, the microplate was shaken and the absorbance was monitored at 600 nm in a microplate reader (GloMax discover, Promega). The assay was performed in triplicate for each bacterial strain. The inhibitory concentrations obtained at MIC were cultured on Müller-Hinton agar (MHA) and incubated for 24 hours at 37°C. The concentrations that did not present microorganism growth were determined as MBC.

Viability and toxicity on cancer cell

Cell culture

SH-SY5Y (human neuroblastoma) was employed as a model to study the anticancer properties of synthetic peptides. SH-SY5Y was cultured in plastic bottles (75 mL) in the presence of high-glucose Dulbecco's Modified Eagle Medium (DMEM). The medium was supplementation with 15 % fetal bovine serum (FBS), 1 % Penicillin/Streptomycin, 4 % HEPES pH 7.4, and 1 % L-Glutamine. Cell line was cultured at 37°C with 5 % CO_2 and the medium was changed every day.

Cytotoxic assay

The evaluation of the cytotoxic properties of the peptides pC-CoaTXIIR2 and pC-CoaTxIIR6 was performed using 3-[4,5-dimethylthiazol-2-yl]-2,5- diphenyltetrazolium (MTT) in the SH-SY5Y cell line. Cells were aliquoted in a sterile 96-well microplate at a density of $7x10^4$ cells/well and then incubated for 4 hours at 37°C in 5 % CO_2. Subsequently, the peptides were added to cells at 50, 75, 150, 250 and 300 µM concentrations and were incubated again at 37°C in 5 % CO_2 for 24 hours. After the second incubation period, the medium was changed to avoid slowing down cellular metabolism and 40 µl of MTT were added to the medium. The microplate was incubated once again for a period of 3 hours at the same conditions. Finally, each well was challenged with 30 µl of 20 % sodium dodecyl sulfate (SDS) solution to dissolve the formazan crystals. Cell viability was measured by spectrophotometry at 600 nm using a microplate reader (GloMax discover, Promega). The antineoplastic drug, Doxorubicin, was used as positive control at 50 µM; while as a negative control, cells growing in supplemented medium were used. The assay was performed in triplicate and results were reported as inhibition of cell viability.

Statistical analysis

The results were analyzed using the GraphPad Prism 8 statistical package, applying the two-way analysis of variance (ANOVA) and Tukey's test. The significance considered was $p<0.05$.

RESULTS

Synthesis and characterization of peptides

Analogues, pC-CoaTxIIR2 y pC-CoaTxIIR6, were synthesized as C-terminal amides by standard solid-phase peptide synthesis (SPPS). The crude peptides were purified by RP-HPLC, obtaining a purity of 92.5 % for pC-CoaTxIIR2 and 97.3 % for pC-CoaTxIIR6 (Figure 2). In addition, the samples were analyzed by MALDI-TOF MS, the first peptide showed a *m/z* signal of 1741.83 Da and the second peptide of 1853.93 Da (Figure 1). The observed masses were similar to the theoretical masses obtained in the *in silico* analysis.

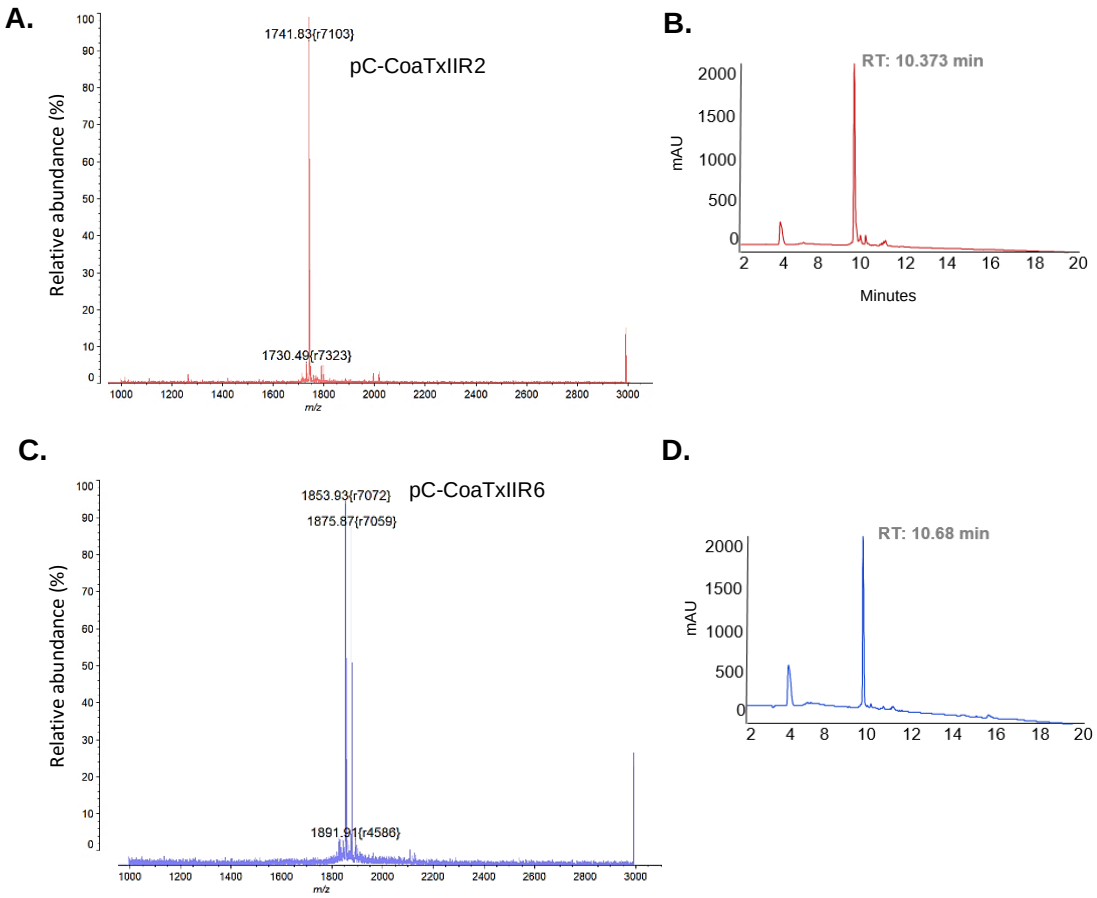

Figure 1 . RP-HPLC profile and mass spectra of analogue peptides.

A) pC-CoaTxIIR2 has a m/z = 1741.83 Da, B) retention time of 10.373 min and 92.5 % purity. C) pC-CoaTxIIR6 has a m/z = 1853.93 Da, D) retention time of 10.68 min and 97.3 % purity.

Bioinformatic analysis

PepCal and PepDraw platforms predicted same length and charge for both phospholipase A_2-derived peptides (Table 1). However, synthetic products slightly differ in molecular weight, pI and hydrophobicity due to their particular lysine and arginine compositions. Consequently, pC-CoaTxIIR6 has the highest isoelectric point (pI = 12.19) and the lowest hydrophobicity (+13.38 kcal/mol) due to changing lysine residues to arginine residues.

Table 1. Physicochemical properties of synthetic peptides calculated by PepDraw and PepCalc tools.

Peptide	Sequence	Length	Molecular weight	Charge	pI	Hydrophobicity [kcal/mol]
pC-CoaTxIIR2	KKYRIYPRFLCKK–NH_2	13	1742.015	+6	10.71	+17.34
pC-CoaTxIIR6	RRYRIYPRFLCRR–NH_2	13	1854.039	+6	12.19	+13.38
pC-CoaTxII	KKYRIYPKFLCKK–NH_2	13	1713.028	+6	10.14	+18.33

Computational analysis predicted the antibacterial, anticancer and cell-penetrating activities of analogue peptides (Supplementary material, Table 2S). On the other hand, alignments performed in Clustal Omega demonstrated the peptides are similar to effective antibacterial and anticancer peptides previously evaluated in other studies [15,20,25]. Most of these peptides are short chains consisting of cationic and hydrophobic amino acids identified in the alignment by purple and red colors, respectively (Figure 2). Finally, the secondary structure bioinformatic tools predicted that the peptides have a randomly coiled structure.

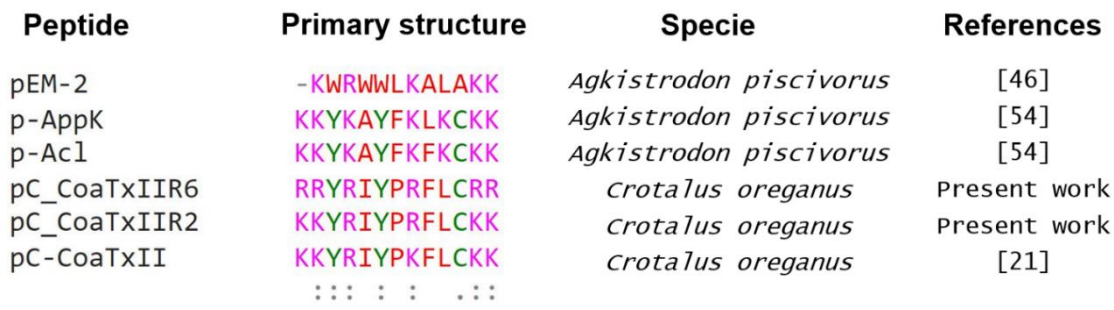

Figure 2. Multiple sequence alignment of synthetic peptides with antibacterial and anticancer peptides inspired by PLA_2s from snake venom**.**

Hemolytic assays

Three online bioinformatic tools were employed to predict the hemolytic character of analogues (pC-CoaTxIIR2 and pC-CoaTxIIR6), template (pC-CoaTxII), toxic (Cupiennin1d) and non-toxic (pBmje). HemoPI indicated that both phospholipase A_2-derived peptides are probably 50 % toxic to erythrocytes. In contrast, HemoPred and HLPpred-Fuse suggested that the synthesized fragments present poor membranolytic activity (Supplementary material, Table 1S). *In vitro* hemolytic assay corroborated that pC-CoaTxIIR2 and pC-CoaTxIIR6 exhibited toxicity to red blood cells (Figure 3). The positive control (2 %Triton X-100) represented 100% erythrocyte lysis. Both analogue products caused approximately 1-11% erythrocyte disruption at the highest peptide concentration tested. However, pC-CoaTxIIR6 peptide caused higher lysis to the RBC membrane, in contrast to pC-CoaTxIIR2. Therefore, erythrocyte lysis is not a limiting factor for the possible application of the synthetic peptides.

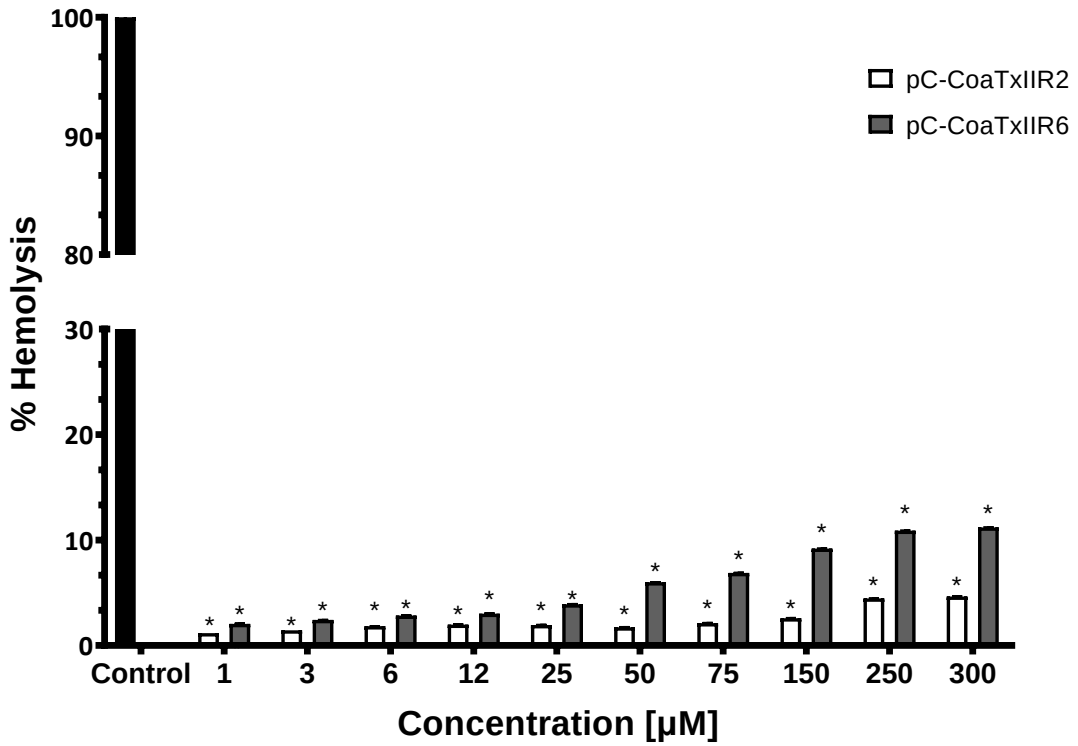

Figure 3. Hemolytic activity of pC-CoaTxIIR2 and pC-CoaTxIIR6.

(*) The action of peptides to red blood cells presented significant differences with respect to the positive control (Triton X-100), determined by ANOVA analysis ($p < 0.05$).

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) assays

In vitro results coincided with the predictions of the bioinformatics tools. pC-CoaTxIIR6 was the most bioactive peptide against *S. aureus* (50 µM) and *E. coli* (75 µM). In contrast, the analog pC-CoaTxIIR2 inhibited bacterial growth at *S. aureus* (75 µM) and *E. coli* (250 µM) concentrations. On the other hand, pC-CoaTxIIR2 and pC-CoaTxIIR6 exhibited MBC values for *E. coli* of ≤ 300 µM and 150 µM, respectively; while the synthetic peptides were effective against *S. aureus* strain at the concentration of 75 µM.

Table 2. Minimum Inhibitory Concentrations (MIC) and Minimum Bactericidal Concentrations (MBC) of analogue peptides

Peptide	MIC (µM)		MBC(µM)	
	E. coli ATCC 25922	*S. aureus* ATCC 25923	*E. coli* ATCC 25922	*S. aureus* ATCC 25923
pC-CoaTxIIR2	250	75	≥ 300	75
pC-CoaTxIIR6	75	50	150	75

Viability and toxicity on cancer cell

The *in vitro* test corroborated the anticancer predictions of peptides. The two venom toxin-derived peptides demonstrated cytolytic activity against SH-SY5Y adherent cell line in all concentrations tested (Figure 4). pC-CoaTxIIR2 has higher cytotoxic effect on neuroblastoma cells than pC-CoaTxIIR6, since the effective concentrations are shown to be EC_{50} = 371.2 µM ± 1.26 and EC_{50} = 741.9 µM ± 0.65, respectively. The percentages of viability cell against peptides analogues at concentration of 300 µM were around 50-60 %. On the other hand, the percentage of viability cell against the positive control (doxorubicin, 50 µM) was approximately 40%. Thus, the cytotoxic effect of both venom toxin-derived peptides is low, in contrast to the drug concentration and the higher concentrations of other antitumor peptides. However, peptides and drug are significantly different from the negative control that represents tumor cells without treatment, corresponding to 100 % cell viability.

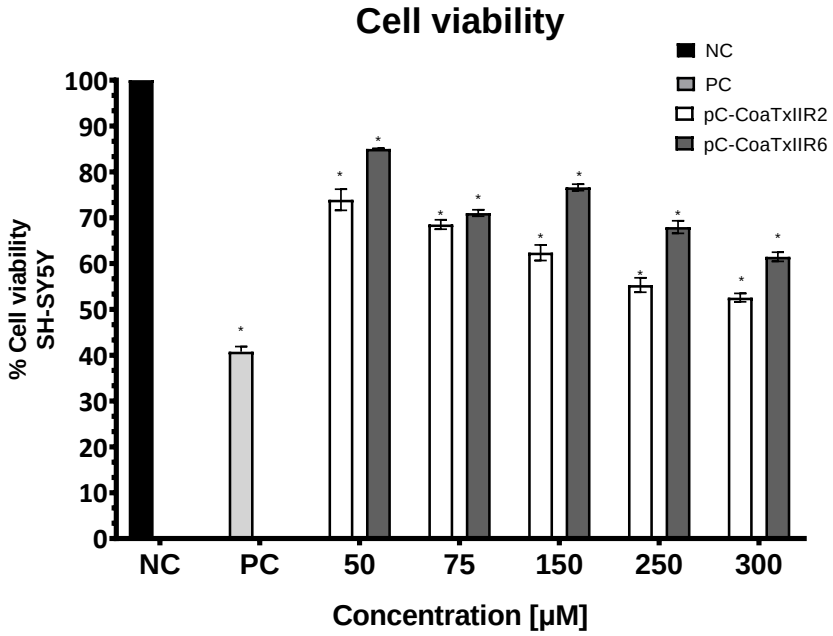

Figure 4. Cytolytic effect of peptides pC-CoaTxIIR2 and pC-CoaTxIIR6 against the SH-SY5Y tumor line.

(*) Positive control (PC, Doxorubicin) and antitumor activities of peptides presented significant differences with respect to the negative control (NC), determined by ANOVA analysis ($p < 0.05$).

DISCUSSION

Peptides are an innovative strategy for the design of pharmacological prototypes due to their diversified biological activity, high affinity and selectivity [26]. Excellent examples are peptides characterized from snake venom that have demonstrated potential antiparasitic [18], antibacterial [27] and antitumor [19] effects. Thus, due to the shared characteristics of peptides, biomedical advances have focused on the evaluation of their multifaceted properties [15,19,28]. From this recognition, it has been shown that the composition of cationic and hydrophobic residues in peptides contributes to the activity against different types of pathogens [18–20].

According to the strategies of peptide therapy based on the evaluation of dual antibacterial and antitumor effects [19], we evaluated two synthetic peptide analogs of the pC-CoaTxII peptide isolated from the C-terminal region of a Lys49-PLA_2. The template molecule was synthesized from a toxin (CoaTxII) with bactericidal activity [13]; thus, pC-CoaTxII conserved and increased the ability to penetrate membranes of

bacterial pathogens [20]. The results obtained corroborate the multifaceted potential of the analogue peptides, since they reproduced lytic activity against bacteria and also presented cytotoxic effects on tumor cells.

Previous studies have reported that hemolytic activity is associated with antimicrobial activities, which limits their application in clinical approaches [29,30]. Antimicrobial peptides (AMPs) such as Melittin (HC_{50} = 1.5 μM) [31], MG-H1 (HC_{50} = 2.9 μM) [32,33] and Cupiennin1d (HC_{50} = 14.5 μM) [31], are specific examples of erythrocyte disruption at minimal inhibitory concentrations of bacterial growth. Nonetheless, higher concentrations of pC-CoaTxIIR2 and pC-CoaTxIIR6 showed low toxicity to human red blood cells. Similarly, the PLA_2-derived peptides pCergo, pBmTxJ and pBmje have shown low toxicity [17], which comes into agreement with the findings for pC-CoaTxIIR2 and pC-CoaTxIIR6. Also, the *in vitro* results agree with the predictions obtained from bioinformatics tools, suggesting that they are useful estimators for the pre-selection of non-hemolytic pharmacological models [34,35].

Cationic peptides derived from the C-terminal region of a PLA_2 are characterized by their affinity to interact with the polar heads of phospholipids of pathogenic bacterial membranes [36,37]. Given this background, the analogues showed activity against *S. aureus* and *E. coli*. Similar to our findings, the pEM-2 peptide isolated from Lys49-PLA_2 demonstrated the formation of electrostatic bonds using artificial coatings similar to bacterial membranes [25]. Nevertheless, the bactericidal activity of pC-CoaTxIIR2 and pC-CoaTxIIR6 was lower than other peptides possessing similar charge [17,20]. In this context, it is indicated that the analogous fragments were less active than the template peptide, pC-CoaTxII (net charge = +6, hydrophobicity = +18.33, molecular mass = 1713.02759) [20]. This suggests that the conservation of lysine residues in the primary structure of pC-CoaTxII might contribute in its hydrophobic interactions to hydrolyze the membrane of microorganisms [25,38,39]. Therefore, the C-terminal segment of PLA_2s Lys49 has the ability to recognize and neutralize lytic action against target cells [40]. However, this could be explored by future research.

In addition, the peptides inspired on Lys49-PLA_2 exhibited different activity against *S. aureus* and *E. coli*. The bacterial inhibition of the synthetic molecules was higher for *S. aureus*. Similarly, the bacteriocin peptide showed higher antibacterial activity for Gram-positive strains [41]. Lopez et al. [41] suggested that the simple composition of a peptidoglycan layer in this type of microorganisms favors the permeabilization of the peptides. Regarding the individual activity of the peptides, pC-CoaTxIIR6 showed higher

activity on *E. coli* than pC-CoaTxIIR2. Previous studies indicate that the biological potential may vary depending on the cationic residues that constitute the sequence [28,38]. Accordingly, Cutrona et al. indicated that the substitution of lysine residues by arginine in the peptides: parisin, BF2 and DesHAP1 increased the antibacterial potential, because the guanidine group, present in arginine, better dissipates the positive charge and facilitates electrostatic interactions with the bacterial plasma coat [42]. It has also been shown that arginine-rich sequences exhibit better lytic properties than polylysine fragments [43,44]. In this context, the higher activity of pC-CoatxIIR6 is closely dependent on its high affinity and specificity to enter pathogenic cells, in contrast to pC-CoaTIIR2 [41,45].

Similar to bacteria, the membrane of various tumor cells is rich in anionic phospholipids [8,23,46]. Consequently, antimicrobial peptides inspired by the C-terminal regions of PLA_2 have been shown to act as promising multifaceted effectors against cancer cells [19,36,38]. Due to the plasma components of pathogens, anticancer peptides (ACPs) have more affinity towards neoplastic cells rather than normal eukaryotic cells [47]. Studies by Dennison et al. and Araya et al. have shown that positive amino acids in PLA_2-derived peptides are responsible for selective toxicity to neoplastic cells [16,36]. In this context, MTT assays were performed to evaluate the cytolytic activity of the peptides. Although, the synthetic products reduced the metabolic activity of neuroblastoma cells, the estimated effective concentrations were higher than the tested concentrations, pC-CoaTxIIR2 (EC_{50} = 371.2 µM, hydrophobicity = +17.34 kcal/mol) and pC-CoaTxIIR6 (EC_{50} = 741.9 µM, hydrophobicity = +13.38 kcal/mol). Ghasemi and coauthors indicate that the metabolic activity of cells subjected to the MTT assay could be erroneously be interpreted, affecting in the representation of cell viability [48]. Despite this, the pathogenic selectivity and lack of hemolytic activity of the peptide analogs demonstrate promising effects that may be enhanced by synergism with other pharmacological strategies [49,50].

Nonetheless, there were significant differences in the cytotoxicity of each peptide, where pC-CoaTxIIR2 inhibited tumor cell growth more than pC-CoaTxIIR6. In this case, pC-CoaTxIIR2 exhibits structural similarities to other homologous anticancer peptides from the C-terminal regions of Lys49-PLA_2. Characterized peptides from the venom of *Agkistrodon piscivorus piscivorus* (p-AppK) [15] and *Bothrops aspe*r (pEM-2D) [19], synthesized from the C-terminal segment of the phospholipase A_2 homologue, are examples of lysine-rich antineoplastics that are functional against solid and liquid tumor

lines and *in vivo* assays. The difference in the primary conformation of the peptides evaluated could have an important impact on the efficacy of the biological functions [19,36,38,51]. Since it affects the affinity of the sequences for chemical, structural and biophysical factors of cancer cells [17].

In addition, it has been reported that the hydrophobicity of peptides could also be involved in the membrane permeabilization and anticancer effect of ACPs [23,52,53]. This is because infected cells undergo overexpression of phosphatidylserine and O-glycosylated mucin that increases negative charges and microvilli on the plasma surface, reducing their fluidity [8,22,47]. For example, Huang et al. indicated that small hydrophobicity increases in L6A/L17A (IC_{50} > 83.6 µM) and A12L/A20L (IC_{50} = 2 µM) peptides enhanced the anticancer activity of the sequences against HeLa cells [47]. This suggests that the high hydrophobicity could also influence the antineoplastic potential of the peptide to cause apoptosis or necrosis of tumor cells [47,52,53]. Furthermore, research has shown that this physicochemical property is also related to the secondary structure of peptides influences plasma interaction [47]. In this case, it has been suggested that inhibitory activities could be due to the ability to adopt different secondary structures that activate the potential [54,55]. To illustrate this, the cecropin-derived peptides which despite having a random structure, adopts the α-helix conformation upon interaction with neoplastic cells and exhibits successful anticancer potential against leukemic lines [54]. Thus, it is proposed that the enhanced hydrophobicity of pC-CoaTxIIR2, in contrast to pC-CoaTxIIR6, improves binding to membrane receptors of infected cells, and could also benefit in the adaptability of the active secondary structure of the peptide.

On the other hand, it has been reported that the affinity of Lys49 PLA_2-derived peptides to drive tumor cell lysis may depend on characteristics other than physicochemical parameters [19]. Since it has been shown that these synthetic molecules can interact with vascular endothelial growth factor receptor-2 (VGEF) of cultured cells, contributing to the inhibition of the vascular endothelial growth factor receptor system of oncogenic cells [56]. This possibility suggests that cationic peptides of the C-terminal region of Lys49 PLA_2 could function as promising antineoplastic pharmacological prototypes to treat tumor angiogenesis [56,57].

Taking together, the poor hemolytic effect and considerable antibacterial and anticancer activity of pC-CoaTxIIR2 and pC-CoaTxIIR5 evidenced the functionality of the snake

toxin-inspired synthetic peptides. However, the selectivity of the synthetic molecules was different for each pathogenic cell type. The literature points out that the peptide analogues and other functional peptides against the diseases of interest, such as: p-Acl [15], myotoxin II-(115-129)-peptide [27], and pepBthTX-I [58] are made up of cationic amino acids. Nevertheless, the composition of the existing positive residues in the sequences are the main difference between the products derived from Lys49-PLA_2 [43,44]. The arginine substitution in pC-CoaTxIIR6 contributed significantly in its antibacterial potential [42,43]. Arginine has been shown to favor interaction with microorganism membranes and thus facilitate permeabilization [42]. Despite this, cell viability results indicate that the conservation of lysine in the primary structure allows the maintenance of the lytic activity exhibited by the C-terminal regions of Lys49 PLA_2 [15,38]. Therefore, the affinity and specificity of the peptides needs to be explored, together with the determination of their mechanisms of action.

CONCLUDING REMARKS

In summary, the results evidence the multifaceted effects of peptides inspired by the C-terminal region of Lys49 PLA_2 against bacterial infections and cancer. Furthermore, it is speculated that the effectiveness of these lytic activities depends on the presence of different cationic residues. In this case, arginine replacement contributed in the inhibition of bacteria; however, it did not cause the same effect in tumor cells, since the conservation of lysine favored the cytotoxic effect. Thus, amino acid substitution is a promising strategy for the design and development of therapeutic candidates. Finally, it is proposed that future research is needed to evaluate and elucidate the mechanisms of lytic action of the fragments on pathogens.

DECLARATION OF COMPETING INTEREST

The authors declare no interest conflict.

APPENDIX A. SUPPLEMENTARY DATA

Table 1S. Hemolytic predictions of phospholipase A_2-derived peptides, template peptide, toxic peptide and non-toxic peptide. *In silico* tools indicated ranges between 0 and 1.

Peptide	HemoPi	HemoPred	HLPpred-Fuse
pC-Coa-TxIIR2	0.50	-	0.155
pC-Coa-TxIIR6	0.49	-	0.176
pC-Coa-TxII	0.48	-	0.185
Cupiennin1d	0.82	+	0.960
pBmje	0.49	-	0.085

1 = hemolytic and 0 = non-hemolytic activity; - = hemolytic activity, + = non-hemolytic activity

Table 2S. Potential predictions of analogues peptides inspired on Lys49 PLA_2s. Computational tools predict values at range between 0 likely non active and 1 likely active.

Peptide	Anticancer activity		Antimicrobial activity		CPP activities
	AntiCp	**AcPred**	**AMP Scanner**	**AMPfun**	**BChemRF-CPPred**
pc Coa-TxIIR2	+	0.994	0.997	0.967	0.877
pc Coa-TxIIR6	+	0.940	0.998	0.964	0.962
pEM-2	+	0.992	1.0	0.985	0.915
p-AppK	+	0.994	0.996	0.963	0.864

+ = cytolytic peptide, - = non-cytolytic peptide

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