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Bothrops moojeni snake peptide cyclodextrin complex: production, characterization and *in vitro* evaluation

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ABSTRACT

The BmooMPα-I moojeni snake peptide was studied aimed to increase the therapeutic use as antitumor agent. Complexes were prepared and its physical characteristics were determined: particle size and Zeta-potential (ζ), antiproliferative and cytotoxic effects. BmooMPα-I peptide associated to Hp-βCD was able to change the cell surface parameters, such as ζ-potential which was negative. Hp-βCD:BmooMPα-I and BmooMPα-I alter the ζ of cells in contact with peptides and its association compound shown negative charge, that no favor the interaction with biological surfaces. In comparison the pure peptide as biocidal of epidermoid Carcinoma cells A431 or epithelial cells rather the fibroblast, the BmooMPα-I / Hp-βCD resulted in significant inhibition of Caco-2 human epithelial cancer cells (31.2 µg/mL after 24h), and Human Epidermoid Carcinoma cells A431(3.9 µg/mL after 24h), in a more efficient manner than peptide alone ($p < 0.001$), and was able to alter the degree of hemolysis. Next, the Lactate Dehydrogenase was examined to know the peptide cytotoxic effects and showed lower cytotoxic effects on EC50 for fibroblastic cells. Based on the BmooMPα-I /Hp-βCD properties observed and its ability of inhibiting the proliferation of epithelial cells, this study highlight that this compound is a biocidal with high potential as an anti-tumoral agent.

Keywords: *BmooMPα-I, peptide, hydroxypropyl-beta-cyclodextrin, Human Epidermoid Carcinoma cells, slow delivery*

1. INTRODUCTION

Snake venoms are the complex mixtures of biologically active proteins, peptides, enzymes, and organic and inorganic compounds [1]. Many active principles produced by animals, plants and bacteria have been studied in the development of new drugs for the treatment of diseases such as thrombosis, cancer and AIDS [2-5].

These active principles are peptides and proteins and have been used as bio-pharmaceuticals since the discovery of insulin in 1922, mainly due to their high activity, specificity and effectiveness compared with more conventional drugs [6-8]. The increasing evidence of the therapeutic potential of these macromolecules has led not only to the production of these compounds in large quantities, but has also attracted the interest of the scientific community [4, 9]. In this context Bernardes et al. [10] study the new peptide BmooMPα-I, a proteinase isolated from the venom of Bothrops moojeni. This enzyme has a molecular mass of about 24.5 kDa and belongs to snake venom metalloproteinases (SVMPs). BmooMPα-I may be of medical interest because it is a defibrinating agent, which could be developed as a therapeutic drug to prevent and treat thrombotic disorders.

For cancer treatment, there is great interest in drug design, providing structural templates for the study of new molecules or cellular mechanisms [11]. Some of these proteins or peptides and enzymes isolated from snake venom when evaluated may bind specifically to cancer cell membranes, affecting the cell migration and proliferation. Since, integrins are important in cell adhesion, cell migration, tissue organization, cell growth, hemostasis and inflammatory responses, so they are in the study for the development of drugs for the treatment of cancer. Snake venom integrins are the low molecular weight molecules with different structure, potency and specificity initially isolated from viperid [8, 12]. Some in vivo assays showed antineoplastic activity against induced tumors in mice. In human, both the crude venom and isolated enzymes revealed antitumor activities in preliminary assays, with measurable clinical responses in the advanced treatment phase. However, research on animal venoms has revealed their high toxicity on tissues and cell cultures, both normal and tumoral [1, 13].

For development a medical formulation with this snake venom peptide, the cyclodextrins were proposed as a drug delivery carrier. Cyclodextrins have an ability to form reversible complexes with drugs, without alteration of their physical, chemical, and biological properties once the guest molecule has been slowly released from their cavity [14-16]. Here we aim to develop a strategy to combat tumoral cells and to improve cellular selectivity by the formation of cyclodextrin-snake venom peptide compound. It was study the BmooMPα-I compounds' ability to inhibit epidermoid cells tumor and was determinate their physico-chemical and toxicological properties.

2. EXPERIMENTAL SECTION

2.1 Isolation of BmooMPα-I peptide. BmooMPα-I peptide was purified according to method described by Bernardes et al.[10]. Crude venom of B. moojeni (200 mg) was dissolved in 50mM ammonium bicarbonate buffer (pH 7.8) and clarified by centrifugation at 10,000g for 10 min. The supernatant solution was chromatographed on a DEAE-Sephadex column (1.7x15 cm), previously equilibrated with 50mM, pH 7.8, ammonium bicarbonate (AMBIC) and eluted with a concentration gradient (50mM x 0.45 M) of the same buffer. Fractions of 3.0 mL/tube were collected, their absorbance at 280 nm were read and those corresponding to peak D2 were pooled, lyophilized, dissolved in 50mM, pH 7.8, ammonium bicarbonate and applied on a 1 x 100 cm Sephadex G-75 column, previously equilibrated with the same buffer. The fibrinogenolytic fraction (peak D2G2) was lyophilized and applied on a column of heparin agarose (1 x 10 cm), previously equilibrated with 10mM, pH 7 Tris–HCl-5mM CaCl2 and eluted with 10mM, pH 7, Tris–HCl–1M NaCl. The flow rate was 40 mL/h and fractions of 2.0mL were collected. Like many venom enzymes, it is stable at pH values between 4 and 10 and stable at 70 ^{o}C for 15 min.

2.2. Preparation of supramolecular complexes. A complex containing 1:1 molar ratio of hydroxypropyl-beta-cyclodextrin (Hp-βCD): BmooMPα-I was prepared for chemical analysis and biological tests using the freeze-dry method [17]. Briefly, BmooMPα-I obtained by peptide isolation was dissolved in milli-Q water by mechanical stirred. The same process was done with Hp-βCD. The two solutions of BmooMPα-I and Hp-βCD were mixed for 2h hours by mechanical stirred. The resulting solution were then frozen in liquid N2 and freeze dried over a period of 30 h using a FreeZone® 4.5 system under 50 mbar vacuum. The dried powders were stored in desiccators until further evaluation [18].

2.3. Particle Size and zeta potential (ζ). The mean particle size and the size distribution of the nanoparticles (NPs) were determined by photon correlation spectroscopy (PCS) using a Zetasizer 3000 HS (Malvern W Instruments, Malvern®, UK). The determination of the ζ-potential was performed by laser Doppler anemometry (Zetasizer 3000 HS, Malvern Instruments, Malvern®, UK) after dilution with KCl 1 mM. The measurements were performed too in contact with normal fibroblasts 3T3 [19-21].

2.4. Hemolytic Assays. Hemolytic activity is a requirement to be tested for any blood contacting medical device because this assay reflects the toxicity of the drug. The test is based on erythrocyte lysis induced. The method is based on release of haemoglobin, which can be measured spectrophotometrically. The haemolytic activity of of BmooMPα-I and Hp-βCD: BmooMPα-I derived peptide was determined using rabbit erythrocytes as described by Yau et al. [22]. Briefly, the erythrocytes were isolated by centrifugation (3 mL) at 5000 rpm for 5 min at 4◦C.The cells were washed thrice with PBS solution and diluted to 10% haematocrit. The erythrocytes (1%) were incubated with the synthesized peptide at various concentrations (0.8 and 125 μg/ mL) dissolved in PBS for 1 h at 37 ◦C. Then, there were action mixture was centrifuged at 3500 rpm for 5 min and the supernatant was determined at optical density 414 nm in a 96-well U-bottom polypropylene micro titer plate using a UV-spectrophotometer. The PBS solution and TritonX-100 were treated as blank and control, respectively. The BmooMPα-I dilutions were made in PBS pH 7.4 [23].

2.5. Mammalian cell culture. The fibroblast cell line 3T3 (Cell Bank, Brazil) was cultured in Modified Eagle Medium (MEM) containing 4.5 g/L glucose, 2.0 mM L-glutamine, 2.2 M sodium pyruvate, 10.0 mM N-2-hydroxyethylpiperazine N-2-ethanesulfonic acid (HEPES) buffer, 2.0 g/L sodium bicarbonate, 100 U/mL amphotericin–gentamicin, and 10% fetal bovine serum (Sigma®, St. Louis, MO, USA) [24].

The human colorectal carcinoma cell line Caco-2 (ATCC, Rockville, MD, USA) and human epidermoid carcinoma cell line A431 (FIOCRUZ, Belo Horizonte, Brazil) were cultured in DMEM containing 4.5 g/L glucose, 2.0 mM L-glutamine, 2.2 M sodium pyruvate, 10.0 mM HEPES, 2.0 mM non-essential amino acids, 2.0 g/L sodium bicarbonate, 100 U/mL amphotericin–gentamicin, and 10% fetal bovine serum. A431 cells are a model cell line (epidermoid carcinoma).

The cells (Caco-2, 3T3 and A431) were subcultured after reaching confluence as observed under a phase-contrast microscope. A single cell suspension of each type was obtained after trypsinization, and the cells were counted in a hemocytometer (Reichert®, Buffalo, NY, USA). Then cells were allowed to attach to the plates for 48 h in a humidified atmosphere containing 5% CO2 at 37.0 °C [25-26].

2.6. Surface Charge Measurements. The influence of increasing concentrations (0.8-125 μg/mL) of the BmooMPα-I and Hp-βCD: BmooMPα-I on Caco-2 cell superficial charge was determined by zeta potential (ZP) measurements using a Malvern Zetasizer Nano® and the Laser Doppler Velocimetry technique. The experiment was conducted at 25 ºC with a disposable cuvette (DPS1060). The reason of compared the influence of peptide and peptide-cyclodextrin on Caco-2 cell culture was made to study the membrane integrity and interactions with the peptides. The interaction membrane peptide was able to change de membrane charge that is an important indicative of the relation cell-drug.

BmooMPα-I and Hp-βCD: BmooMPα-I stock solutions were prepared in 100 mM phosphate buffer, pH 7.4 at a 10-fold higher concentration than needed. Separately, a Caco-2 cell suspension at 105cell/mL was prepared in 100 mM phosphate buffer, pH 7.4 from a fresh cellular culture. Next, 100 μL of each standard solution of BmooMPα-I or Hp-βCD: BmooMPα-I compounds were added to 900 μL of Caco-2 cell suspension. The suspension was transferred to a disposable cuvette and the zeta potential measurement was made in triplicate.

2.7. Lactate Dehydrogenase (LDH) Assay. Dehydrogenase-based assays reflect cell conditions with more sensitivity than the other assays because they depend on several elements including dehydrogenase, NAD (H), NADP(H), and mitochondrial activity [27-28].

To prepare samples for the LDH assay, cells of passage number 5-6 were used. One hundred microlitre of cells at a density of 5 × 105 cells/mL (DMEM High glucose containing 10% FBS) were seeded in each well of 96-well plates and grown for 48 h before tested compound exposure. The cells were washed with PBS three times and dosed with 125 to 0.8 μg/ mL, of the

compounds in DMEM red phenol free. After 24 h exposure, the 96-well plates were shaken briefly to homogenize the released LDH in the cell culture medium and the medium was transferred to 1.5 mL micro centrifuge tubes and were centrifuged at 12,000 × g and 4 °C for 15 min to remove any cell debris and NPs. One hundred microliters (100 µl) of each sample was added to the substrate solution and the absorbance at 440 nm was measured using a spectrophotometer (Thermo Scientific Multiskan® Spectrum, Vantaa, Finland) [26]. Pure peptide and peptide-cyclodextrin compound EC50 from different cell types were determined by nonlinear regression using the sigmoidal dose-response equation.

2.8. Cell Proliferation (MTT Assay).

After reaching confluence, each cell type was exposed 24 h to a series of two-fold dilutions of BmooMPα-I or Hp-βCD: BmooMPα-I compounds in the range of 125 µg/mL to 0.8 µg/mL. Compound cytotoxicity was assessed using an MTT assay as described by Mosmann [25-26]. The absorbance data were converted to viability percentages based on the control, which contained culture medium only. The proliferation percentages were compared by a two-way analysis of variance (ANOVA) test, followed by a Bonferroni test, using the statistics software GraphPad Prism 5.0. Pure BmooMPα-I and Hp-βCD: BmooMPα-I compound EC50 from different cell types were determined.

The effective concentration 50 (EC50) was defined as the value when the response is halfway between minimum and maximum response in a dose-response curve, which in this case was the compound concentration required to achieve 50% cell death compared to the control [23-24]. The significance of the difference between the EC_{50} was calculated using a Student's t-test. Statistical significance was considered at $p < 0.05$.

3. RESULTS AND DISCUSSION SECTION

In this study, BmooMPα-I was purified and combined with Hp-βCD to form complexes. The physicochemical properties of both forms were characterized on the results and the complexes shown intermediary or best properties that Hp-βCD and BmooMPα-I.

3.1. Particle measurement and Zeta potential (ζ).

The physicochemical properties displayed by the BmooMPα-I: cyclodextrin and control nanoparticles are depicted in Table 1. The polydispersity index (PDI) is a number calculated from a simple two parameter fit to the correlation data as cumulates analysis. The polydispersity index is dimensionless and scaled such that values $p < 0.05$ are rarely seen other than with latex standards. The various size distribution algorithms work with data that falls between these two extremes. The maximum value is arbitrarily limited to 1.0 [27].

Table 1. Particle size (nm) and zeta potential of BmooMPα-I peptide and Hp-βCD: BmooMPα-I prepared by addition of Hp-βCD (means±S.D. of n=3).

Groups	Particle measurement (nm)	Zeta Potential	PDI
BmooMPα-I	314.6 (+16.7)	-4.65 (+ 0.3)	0.4
Hp-β-Cd: BmooMPα-I	370.3 (+14.9)	-6.49 (+ 0.7)	0.3
Hp-β-Cd	255.7 (+16.3)	-7.14 (+ 0.8)	0.4

The average size of the Hp-βCD: BmooMPα-I nanoparticles remained of 370.3 nm. These values were observed with the controls nanoparticles 314.6 and 255.7, respectively for BmooMPα-I and Hp-βCD. The ζ-potential values was slightly negatives: BmooMPα-I (-4.65); Hp-βCD: BmooMPα-I (-6.49) and Hp-βCD (-7.14) ($p < 0.05$). The zeta potential value of the compound Hp-βCD: BmooMPα-I was near to the Hp-βCD zeta potential than BmooMPα-I evidenced the interaction peptide-cyclodextrin ($p < 0.05$).

3.2. Hemolytic effect.

As shown in Figure 1, the hemolysis generated by BmooMPα-I, associated with Hp-βCD was not more than 50% at 15.6 - 62.5 µg/mL. Similar behaviour was observed with BmooMPα-I alone, which generated the same hemolysis at ≥15.6 µg /mL ($p < 0.05$). The association with cyclodextrin did not affect the cytotoxicity of the peptides on erythrocytes culture compared with pure peptide.

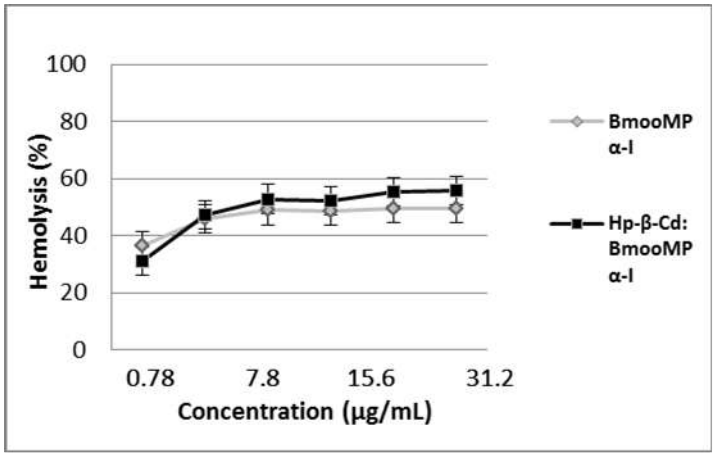

Figure 1. Percentage of hemolysis of rabbit erythrocytes generated by increasing concentrations of BmooMPα-I, Hp-βCD: BmooMPα-I and Hp-βCD 126x69mm (300 x 300 DPI).

3.3. Surface Charge.

The fibroblasts exhibited a baseline negative ζ- potential of approximately -16.9 mV (+ 1.58). However, upon titration with increasing concentrations of Hp-βCD : BmooMPα-I and BmooMPα-I (Fig. 2B and 2C), the surface ζ -potential increased to values of approximately -5 mV after 24h of contact with Caco-2 cells and show low changes after 48h.

Furthermore, the titration of increasing concentrations of Hp-βCD alone on fibroblasts (Fig. 2C) did not have a substantial effect on the ζ- potential for the lower concentration tested: 7.8 and 0.78 µg/ mL, which remained approximately -10 mV at more higher concentrations the values increased to values of approximately -6 mV (125; 62.5; 31.2 µg/mL) ($p < 0.05$).

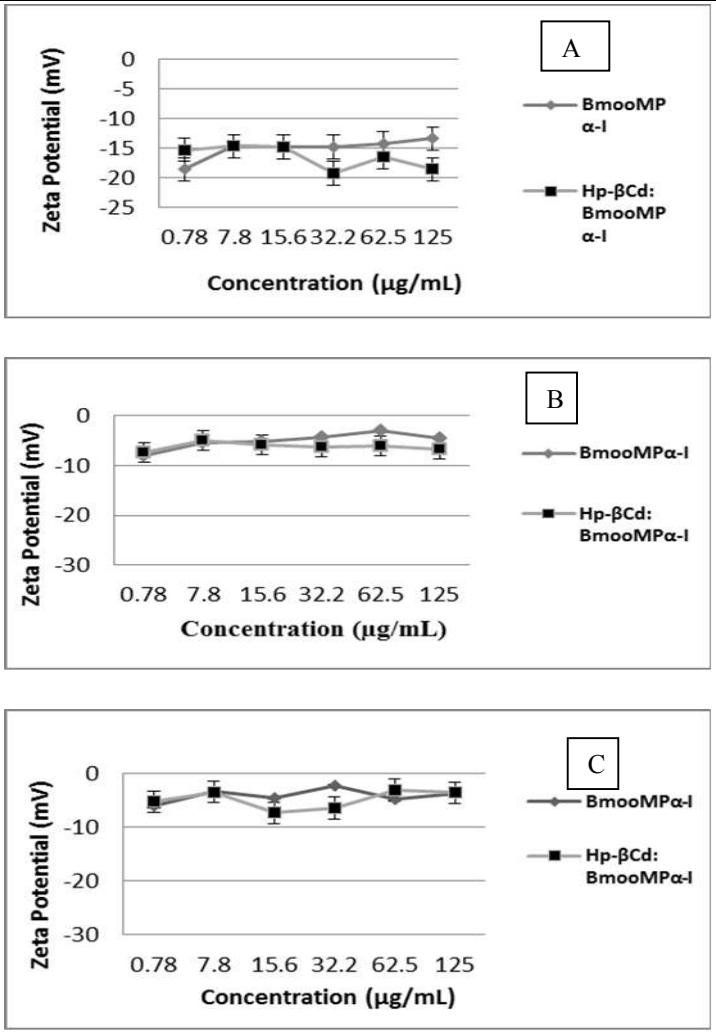

Figure 2. Influence of increasing concentrations of the BmooMPα-I , Hp-βCD: BmooMPα-I and Hp-βCD on Caco-2 cell superficial charge determined by ζ -potential immediately, 24 and 48h using a Malvern Zetasizer Nano ZS and the Laser Dopple Velocimetry technique. (A) T=0 (B) T=24h (C) T= 48h (300 x 300 DPI).

3.4. LDH Release.

Analyzing Fig. 3 one can observe that the highest values of LDH enzyme associated to the complex Hp-βCD:BmooMPα-I are evidencing the cytotoxicity of the pure peptide when compared with the complexed peptide.

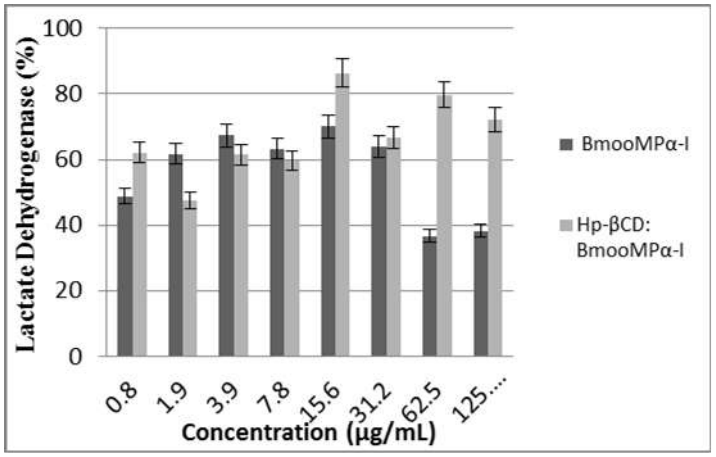

Figure 3. Comparison of the effect of BmooMPα-I, Hp-βCD:BmooMPα-I and Hp-βCD by LDH assay in fibroblasts cells after 24h of exposure; data presented as percentage of control (n=3) ± standard error mean.

3.5. Proliferation Assay.

We next assessed the cell viability of Caco-2, Human epidermoid cancer cells A431 and fibroblasts exposed to a Bothrops moojeni snake peptide and its complex with Hp-βCD (Fig. 4 to 6). The average percent viability of cells treated with these peptide compounds were compared to controls that was cells contained culture medium and cells treated with Hp-βCD.

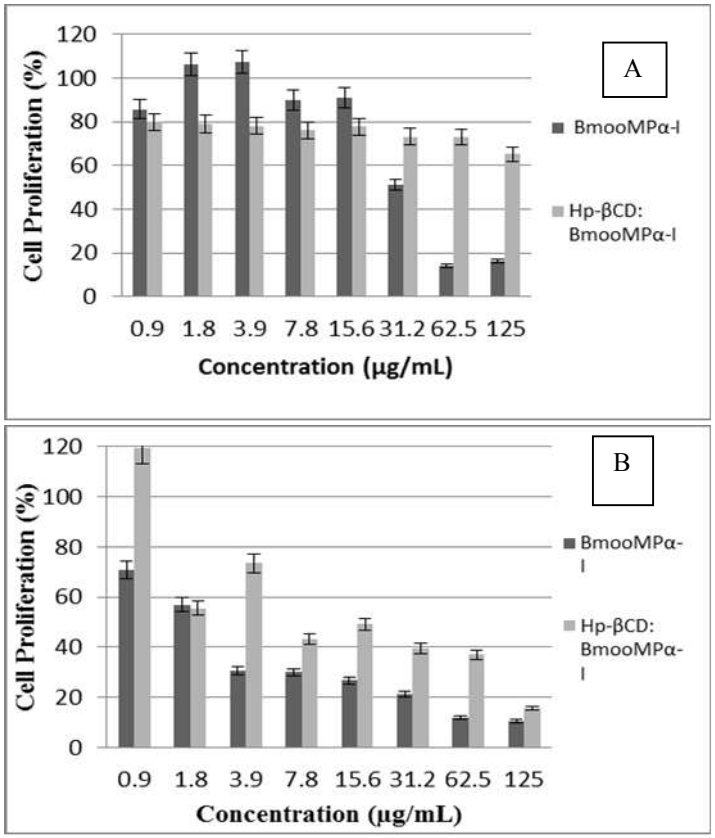

Figure 4. Cell viability/proliferation MTT assay of Caco-2 human cancer cell exposed to (A) 24h and (B) 48h of BmooMPα-I, BmooMPα-I, Hp-βCD : BmooMPα-I and Hp-βCD at 0.8 to 125 µg/ mL. The values are expressed as mean ± SD obtained from three independent experiments carried out in sixplicate.

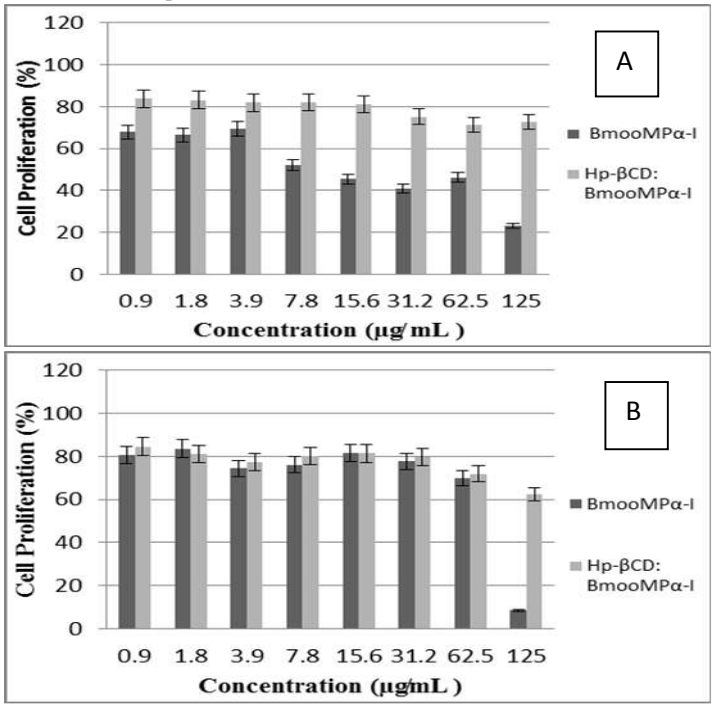

Figure 5. Cell viability/proliferation MTT assay of Human epidermoid carcinoma cells A431 exposed for 24h (A) and 48h (B) to BmooMPα-I, BmooMPα-I, Hp-βCD : BmooMPα-I and Hp-βCD at 0.8 to 125 µg/ mL. The values are expressed as mean ± SD obtained from three independent experiments carried out in six plicate.

In the present study, the lowest concentration of 31.2 µg/mL of BmooMPα-I significantly reduced the proliferation of Caco-2 human cancer cell after 24h $p < 0.05$; Fig. 4A. The effect of the concentration on cytotoxicity of Hp-βCD: BmooMPα-I complex was more expressive after 48h ≥ 62.5 µg/mL ($p < 0.05$; Fig. 4B). The BmooMPα-I was not cytotoxic to Caco-2 cells at concentrations lesser than 31.2 µg/mL and after 24h, but on the time 48h the cytotoxic effect were more severe, the peptides were cytotoxic at 1.9 µg/mL ($p < 0.05$) (Figure 4).

We found that, treatment with 7.8 µg/mL of BmooMPα-I reduced significantly the proliferation of Human epidermoid carcinoma cells A431 ($p < 0.05$; Fig. 5A and Fig 5 B). The cytotoxicity of Hp-βCD: BmooMPα-I was lower than pure BmooMPα-I, and the proliferation reduction effects are more expressive after 48h at a dose of 62.5 µg/mL ($p < 0.05$; Fig.5). The Hp-βCD: BmooMPα-I did not show satisfactory effects on Human epidermoid carcinoma cells A431 after 24h, but the pure peptide show severe cytotoxic effect for these cells at 62.5 µg/mL. The Hp-βCD samples show absence or slow cytotoxic effects on the tested concentrations (Figure 5). The BmooMPα-I was not cytotoxic to A431cells at a concentration lesser than 3.9 µg/mL after 24h.

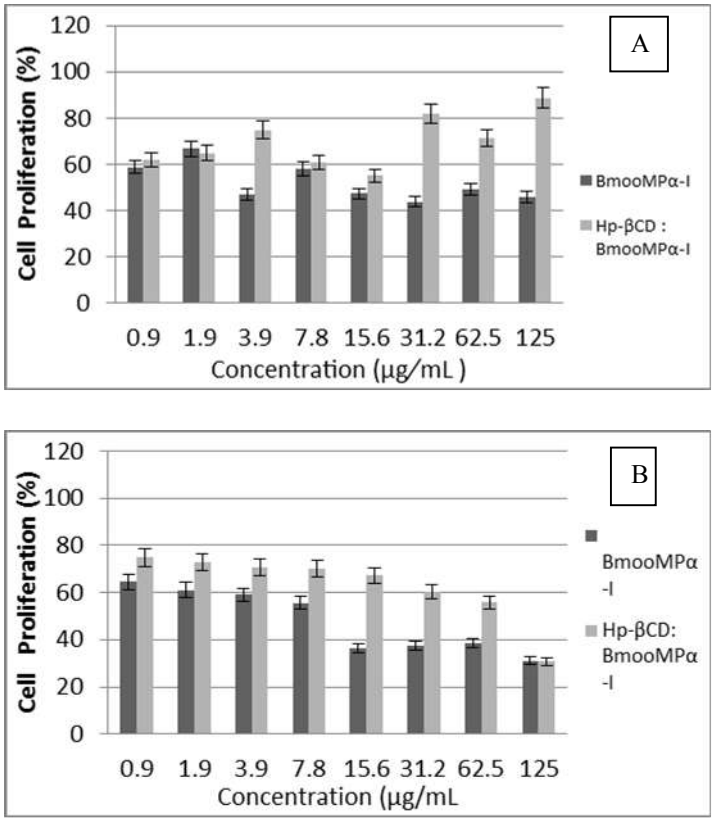

Figure 6. Cell viability/proliferation MTT assay of fibroblasts exposed for 24h (A) and 48h (B) to BmooMPα-I, BmooMPα-I, Hp-βCD : BmooMPα-I and Hp-βCD at 125 to 0.9 µg/ mL. The values are expressed as mean ± SD obtained from three independent experiments carried out in six plicate.

To study the proliferation rates of healthy cells in contact with snake venom peptide was assessed the fibroblasts proliferation (Fig.6). Progressive decreases in the concentration of the agent was correlated with parallel increases in fibroblasts cell survival, for BmooMPα-I. Hp-βCD: BmooMPα-I after 24h.

The cytotoxicity of Hp-βCD: BmooMPα-I was lower than pure BmooMPα-I, and the proliferation reduction effects are more expressive after 48h, 64.8 and 32.8% at a dose of 15. 6 µg/mL ($p < 0.05$); (Fig. 6B).

When assessed the cytotoxic effects of snake venom peptide and its association with cyclodextrin on Caco-2 epithelial cells both were significantly cytotoxic at a concentration of 1.9 µg/ mL after 48h (Fig. 4B). BmooMPα-I exhibited cytotoxic effects against the A431 cells at concentration of 125 and 62.5 µg/mL. In addition, Hp-βCD: BmooMPα-I had a moderate cytotoxic effects for fibroblasts at a concentration of 62.5 µg/mL, and the BmooMPα-I compound exhibited an even greater cytotoxic effect by reducing cell viability at a concentration of 3.9 µg/mL ($p < 0.05$).

The EC50 values of the BmooMPα-I, Hp-βCD:BmooMPα-I and Hp-βCD in Caco-2 cells, A431 cells and fibroblasts are shown in Table 2. The EC50 values of the peptides and compounds with Hp-βCD were greater at 24h than 48h for Hp-βCD: BmooMPα-I, probably it was cause by association with cyclodextrin and the peptide delivery more slowly. The cytotoxic concentrations of these compounds were lower for epithelial cells than for fibroblasts. However, Hp-βCD alone did not decrease a statistically significant in cell viability for both cell types compared to the blank control $p < 0.05$).

Table 2. The effective concentration 50 (EC50) of BmooMPα-I and Hp-βCD: BmooMPα-I in epithelial Caco-2 cells, Human Epidermoid Carcinoma cells A431 and fibroblasts.

Groups	Caco-2 cells (µg/mL)		A431 cells (µg/mL)		Fibroblasts (µg/mL)	
	24h	48h	24h	48h	24h	48h
BMOOPαI	31.2	1.9	15.8	125	15.8	7.8
Hp-β-Cd: BmooMPα-I	3.9	1.9	7.8	125	-	62.5

In the present work, the introduction of the cyclodextrin as a second component in the molecular complex was expected to optimizing the interaction with biological surfaces [28]. Cyclodextrins are neither hydrolyzed nor absorbed in the stomach or the small intestine, making them effective protectors of different classes of guest molecules. Thus, a synergic effect could be reached using cyclodextrins as drug carrier systems for peptides and proteins. In addition, cyclodextrins are able to recognize not only the size but also the shape of peptides and proteins.

These molecules may be too large to be wholly included in the cyclodextrin cavity. However, these guest molecules could present hydrophobic side chains in their backbones, which can interact specifically and form inclusion or association complexes with cyclodextrins [28-29]. The appropriate selection of molecules used for the preparation of the release systems permit the modulation of their physicochemical properties, such as hydrophobicity, surface charge and drug-release profiles [5].

The cyclodextrin was selected on this study because this is used as absorption enhancers. The hydrophilic outer surface of Cd molecules forms a weak interaction with biological membrane [30-31]. Complexation of drugs in cyclodextrin (Cd) has been shown to represent an effective strategy for improving macromolecular drug therapy by stabilizing the guest molecule

against aggregation, thermal denaturation, or degradation [30]. Previous study shown that BmooMPα-I did not induce hemorrhage on mouse back skin when 30 μg of enzyme was intradermally injected [10].

A positive surface charge is considered to be an advantage from the biopharmaceutical viewpoint, as it favors the muco and bioadhesion of the nanoparticles to negatively charged sites on cell surfaces and tight junctions [32-35]. The zeta potential observed on these studies suggested a need to development a formulation with positive charge. This study shown at figure 2 there was a change on the surface charges after 24h changing the cells zeta potential more positive. In this study the included peptide shown a zeta potential like as the cyclodextrin, this fact suggested the interaction of the substance and the position of the cyclodextrin external to the peptide. The snake venom peptide and peptide:cyclodextrin show nanometric size, that advantageous for the absorption penetrability and interaction with tissues.

Diverse biological activities were associated with snake venom peptides. Most of them are associated with haemorrhage or the disruption of the haemostatic system, which is primarily, mediated by the proteolytic activity of the metalloproteinases [36]. Some of these animal and vegetable peptides also have other biological activities that have impacts on cell proliferation, immune induction, cytokine release, chemotaxis and tissue repair [2,3,37,38]. In this work was evidenced the snake venom peptides show antitumoral effect for epithelial cells: A431 and Caco-2. The cyclodextrin capacity to modulated the cytotoxic effects on fibroblasts and tumoral cells was an advance on the snake venom peptides study because this molecules shown severe cytotoxic effects.

The LDH leakage assay was used us a complementally assay to shown cytotoxic effects and study cell integrity. The motivation of the use of LDH and MTT assay on this study was based on the different point assays of these assays. The LDH assay is based on the release of the enzyme into the culture medium, after cell membrane damage whereas the MTT assay is mainly based on the enzymatic conversion of MTT in the mitochondria evidenced membrane integrity and protein synthesis activity respectively [39-40]. The release of LDH from cells in cell culture studies was found to be associated with necrosis as well as apoptosis [39-41] and hence, further investigations are necessary for conclusions about the pathway of cytotoxic effects [40]. *Zhang* &Cui [42] isolated ACTX-6 (98 kDa proteins containing two subunits) from *Agkistrodon acutus* snake venom. The authors found that ACTX-6 could induce cell apoptosis. The induction of the apoptosis manifests the control on the tumour size and number of tumour cells hence establishing the application of apoptotic inducers as vital components for antitumor effect in vitro.

Currently in the LDH assay a membrane leakage caused by the interaction with the snake peptide venom is evidenced. Whereas, the BmooMPα-I shown greater cytotoxic effects for fibroblasts when compare with Hp-βCD: BmooMPα-I. The Hp-βCD did not alter fibroblasts proliferation at tested concentrations. Thus, molecular association cyclodextrin-peptide could be considered as important tool in promoting membrane disruption at early stages of the proliferation of tumor cell [43-44].

On this work was studied the cytotoxic effects by MTT and LDH methods, the difference on the results were justified by the toxicity of the BmooMPα-I on different cellular structure: mitochondria and cell membrane. The LDH assay evidenced the membrane leakage caused by BmooMPα-I treatment and MTT the influence of the peptide on cell proliferation, both show the lesser cytotoxic effects of the peptide-cyclodextrin on fibroblasts.

As shown in Fig. 5, the BmooMPα-I peptide and Hp-βCD : BmooMPα-I have cytotoxic potential and the statistical differences found between the EC_{50} values compounds on Caco-2 cells, let us to conclude that Hp-βCD increases the cellular toxicity of these peptides against this cellular lineage. On the other hand, the molecular encapsulation attenuated the toxicity against mammalian cells. Hp-βCD: BmooMPα-I compounds were not cytotoxic against fibroblasts (Figs. 3 and 4). The ability of some snake venom toxins to cause toxicity is associated with their high specificity and affinity for cell and tissues. In spite of their toxicological effects, several isolated snake venom proteins and peptides have practical applications as pharmaceutical agents [11] and by the selective channel blockers which can be overexpressed in different tumours.

The interference of toxins present in snake venoms occurs often due to unique characteristics of enzyme activity, structure and bonding. In short, toxins, such as metalloproteases and disintegrins of snake are important tools for basic studies of haemostasis, mainly related to platelet function and development and metastasis of tumor cells, as well as have therapeutic potential. These observations were very important to the antitumoral results observed of this study and justify the antitumoral properties. Certainly, advances in research on the biochemical constitution of venoms lead to promising discoveries for antiplatelet therapy (antithrombotic) and antitumor [45-49]. Residues adjacent to the tripeptide sequence Arg-Gly-Asp (RGD), the three-dimensional structure, the structure of the C-terminal region and the pattern of disulfide bonds of disintegrins appear to interfere with the specific binding of these molecules to integrins [45,50,51].

There are studies showing that Bothrops venom induces inhibition of Ehrlich ascites tumour (EAT) growth, accompanied by an increase of mononuclear (MN) leukocytes in all groups inoculated with EAT and/or venom [11]. The previous observations supported this study; disintegrins also possess the ability to inhibit tumour proliferation both in vitro and in vivo. Salmosin, a disintegrin isolate from Korean snake venom, effectively suppressed growth of metastatic tumour as well as solid tumour in mice. This anti-metastatic activity was resulted from blockage of integrin-mediated adherence of αvβ3 integrin mediated proliferation of the melanoma cells [1,52].

The results obtained in this study from the cytotoxicity assays indicate that there are differences between the three cell lines concerning their sensitivity to BmooMPα-I and Hp-βCD: BmooMPα-I. The Caco-2 epithelial cells appear to be more sensitive as indicated by the MTT assay. Changes in the cell surface or the sensitive lysosomal membrane lead to lysosome fragility and other changes that gradually become irreversible [53].

We propose that the cytotoxic activity on tumor cell lineages observed in this work is associated with the interactions between cells and the basement membrane through the degradation of cell membrane proteins and basement membrane components (*e.g.*, fibronectin, laminin, nidogen, type IV collagen).

Blood coagulation proteins (*e.g.,*fibrinogen, factor X, prothrombin) are also targets of their proteolytic activities. Consequently the peptide-cyclodextrin complex obtained here could be further analyzed on in vivo tumor for assess the anti-proliferative effect.

4. CONCLUSIONS

Our results suggested that the BmooMPα-I /Hp-βCD complexes different properties of the pure cyclodextrin and peptide, this change was able to influence the cellular effects *in vitro*. The different responses verify was specific for each cell type, highlighted that the surface charge on this mechanism.

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