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ORIGINAL ARTICLE

Anti-proliferative and cytotoxic activity of pentadactylin isolated from *Leptodactylus labyrinthicus* on melanoma cells

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Abstract Nowadays, the emergence of resistance to the current available chemotherapeutic drugs by cancer cells makes the development of new agents imperative. The skin secretion of amphibians is a natural rich source of antimicrobial peptides (AMP), and researchers have shown that some of these wide spectrum molecules are also toxic to cancer cells. The aim of this study was to verify a putative anticancer activity of the AMP pentadactylin isolated for the first time from the skin secretion of the frog *Leptodactylus labyrinthicus* and also to study its cytotoxic mechanism to the murine melanoma cell line B16F10. The results have shown that pentadactylin reduces the cell viability of B16F10 cells in a dose-dependent manner. It was also cytotoxic to normal human fibroblast cells; nevertheless, pentadactylin was more potent in the first case. The studies of action mechanism revealed that pentadactylin causes cell morphology alterations (e.g., round shape and shrinkage morphology), membrane disruption, DNA fragmentation, cell cycle arrest at the S phase, and alteration of mitochondrial membrane potential, suggesting that B16F10 cells die by apoptosis. The exact mechanism that causes reduction of cell viability and cytotoxicity after treatment with pentadactylin is still unknown. In conclusion, as cancer cells become resilient to death, it is worthwhile the discovery of new drugs such as pentadactylin that induces apoptosis.

Keywords Amphibian · Apoptosis · B16F10 · Cytotoxicity · *Leptodactylus labyrinthicus* · Pentadactylin

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Introduction

Despite the recent progress in cancer therapy, this disease continues being one of the major causes of morbidity and mortality in the world. The word cancer refers to different sorts of diseases, affecting distinct tissues and cell types, characterized by abnormal cellular growth, resulted from genetic mutations (Hoskin and Ramamoorthy 2008).

Although localized cancer can be treated by surgery or radiotherapy, chemotherapy is still the treatment of choice for the most serious cases. Nevertheless, the conventional chemotherapeutic agents, which act against high proliferating cells through nucleic acid synthesis inhibition, induce unwanted side effects. The therapy is still complicated because of the resistance acquired by tumors to chemotherapeutics (Dennison et al. 2006; Hoskin and Ramamoorthy 2008).

Among the different types of cancer, melanoma is a huge public health problem because of its increase of 3–8% per year. It has happened mainly due to an augment of the

exposure to the environmental ultraviolet rays that induce abnormalities in genetic pathways within melanocytes. Early detected cases are usually cured by surgery; on the other hand, advanced melanomas need the use of systemic therapies. Frequently, chemotherapy is done using dacarbazine, fotemustine, and temozolomide; however, they present low response rates. In this way, it is imperative the development of more effective therapies (Thompson et al. 2005).

Recently, the development of treatments based on antimicrobial peptides (AMPs) has emerged as a new strategy to cancer therapy. These peptides have different important roles in the innate immunity of diverse organisms. The use of these molecules is advantageous since AMPs are more selective to the negatively charged membrane of cancer cells than to the membrane of normal cells, show good penetrability in the tissues due to their small size, act rapidly, do not stimulate cell resistance, display synergism with classic drugs, hold a broad activity spectrum, and are capable of destroying primary tumors, besides preventing metastasis (Bhutia and Maiti 2008; Shadidi and Sioud 2003).

A diverse number of organisms produce AMPs; however, the major source of these peptides (e.g., magainins, dermaseptins, gaegurins, aureins, and citropins) is the skin secretion of amphibians. Studies developed during the last decades have shown that a lot of broad spectrum AMPs also exhibit anticancer activity (Pukala et al. 2006). Inside the group of broad spectrum AMPs there is the cationic peptide pentadactylin, first isolated from the South American bullfrog *Leptodactylus pentadactylus*. Pentadactylin is composed by 25 amino acids, presenting moderated amphipacity, and propensity to acquire an α-helical conformation in a membrane-mimetic solvent. This peptide is active against a wide range of Gram-positive and Gram-negative bacteria. Moreover, pentadactylin presents low toxicity to normal eukaryotic cells (King et al. 2005).

The aim of this study was to verify a putative anticancer activity of the AMP pentadactylin isolated for the first time from the skin secretion of the frog *Leptodactylus labyrinthicus* and also to study its cytotoxic mechanism to the murine melanoma cell line B16F10.

Materials and methods

Specimens collection and skin secretion extraction

Adult specimens of *Leptodactylus labyrinthicus* were collected in Luziânia/State of Goiás and maintained in captivity at the University of Brasilia. The skin secretion was obtained by mild electrical stimulation method, diluted in Milli Q water, lyophilized and kept frozen (−20°C) for subsequent use. The animals reassumed their normal behavior a few minutes after the secretion harvesting. This procedure was approved by the Animal Ethics Committee of the University of Brasilia.

Purification of pentadactylin

The freeze-dried secretion (5.0 mg) was dissolved in 0.1% (v/v) TFA/water (200 µL), and subjected to RP-HPLC on a C_{18} column (Vydac 218TP54, 4.6 × 250 mm) equilibrated with 0.1% (v/v) TFA/water (solvent A). After an initial 5-min wash with solvent A, elution was performed at a flow rate of 1 mL/min with a 0–60% linear gradient of acetonitrile containing 0.1% (v/v) TFA (solvent B) during 60 min, then from 60 to 100% of solvent B in 5 min and a final 5 min wash with 100% of solvent B. The absorbance was monitored at 216 nm. All fractions were manually collected, lyophilized, and screened for antimicrobial activity to *Escherichia coli*, and *Staphylococcus aureus*. The fraction containing the peptide of interest was accumulated, and injected onto a C_{18} reversed-phase HPLC column (Onyx monolithic, CHO-7643, 4.6 × 100 mm) equilibrated with 0.1% (v/v) TFA/water. Elution was performed, at a flow rate of 1 mL/min, with a 0–32% linear gradient of solvent B during 5 min, 32–37% of solvent B in 20 min, 37–100% of solvent B in 5 min, and a final 5 min wash with 100% of solvent B. The eluent was monitored by UV absorbance at 216 nm. Fractions were collected and dried in vacuum for subsequent analysis.

Structural analysis

Mass analysis of the purified peptide was performed using an Autoflex II TOF/TOF mass spectrometer (Bruker Daltonik, Bremen, Germany) in the 500–4,000 Da range in the reflection positive mode. The external standard used for MALDI-MS analysis was a mixture of angiotensin II (M_r, 1046.54180), angiotensin I (M_r, 1296.68478), substance P (M_r, 1347.73543), bombesin (M_r, 1619.82235) and adrenocorticotropic hormone fragment 18–39 (M_r, 2465.19834). The isolated peptide was sequenced by automated Edman degradation using an Applied Biosystems 477A sequencer modified as described in Fontes et al. (1998). The amidation at the C terminus portion was confirmed by mass spectrometric analysis of the methylated peptide (Hunt et al. 1986). In summary, an aliquot of the peptide was dissolved in 20 µL of methanolic HCl reagent (10 µL of acetyl chloride in 250 µL of distilled methanol), and incubated at RT for 30 min. Solvent was removed by lyophilization, and the methylated peptide was reconstituted with 20 µL of 0.1% (v/v) TFA in 50% (v/v)

acetonitrile, and then analyzed by Autoflex II TOF/TOF mass spectrometer (Bruker Daltonik, Bremen, Germany) as described above. The amino acid sequence similarity search was done using NCBI non-redundant database through the PSI-Blast algorithm (Altschul et al. 1997) (http://www.ncbi.nlm.nih.gov/BLAST), as well as off-line comparison to other peptides isolated from *Leptodactylus*, using information available in the current literature.

Peptide synthesis

The pentadactylin peptide used in the assays (with amidated C terminus) was synthesized manually according to the standard N^{α}-Fmoc protecting-group strategy (Atherton and Sheppard 1989), using the experimental steps described in Casallanovo et al. (2006) . The side chain protecting group Boc (*t*-butoxycarbonyl) was used for K, *t*But (*t*-butyl) for D, T and E, and Pmc (2,2,5,7,8-pentamethyl-chromane-6-sulfonyl) was used for Q and N. After the coupling of the C-terminal amino acid to 4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxyacetamido-norleucyl-(4-methylbenzhydrylamine) resin (Rink-amide-MBHAR), the successive α-amino group deprotection and neutralization steps were performed in 20% piperidine/dimethylformamide (DMF) for 20 min. The amino acids were coupled at threefold excess using diisopropylcarbodiimide (DIC)/*N*-hydroxybenzotriazole (HOBt) in 50% (v/v) DCM (methylene chloride)/DMF and, if necessary, 2-(1H-benzotriazole-1-yil)-1,1,3,3-tetramethyluroniumhexafluorophosphate (TBTU)/HOBt/diisopropyl ethylamine (DIEA) in 50% (v/v) DCM/*N*-methylpyrrolidone (NMP). After a 2 h coupling time, the ninhydrin test was performed to estimate the completeness of the reaction. Cleavage from the resin and removal of the side chain protecting groups were simultaneously performed with 90% TFA, 5% *p*-cresol and 5% water during 2 h. In this procedure, the crude peptide was precipitated with anhydrous ethyl ether, separated from soluble non-peptide material by centrifugation, extracted into 0.045% (v/v) TFA/H_2O (solvent A), and lyophilized.

The peptide, after dissolution in solvent A, was purified by semi-preparative RP-HPLC on a Shimadzu system (Japan) using a reverse phase C_{18} column with a linear gradient of 10–40% of solvent B (0.036% (v/v) TFA/acetonitrile) over 90 min. The flow rate was 5 mL/min. UV detection was carried out at 216 nm. The impurities derived from the synthesis process were removed by analytical RP-HPLC, using a C_{18} column (Vydac 218TP54, 4.6 × 250 mm), and UV detection at 216 nm. The identity of the peptide was confirmed by mass spectrometry using a Autoflex II TOF/TOF (Bruker Daltonik, Bremen, Germany) and amino acid analysis (Shimadzu model LC-10A/C-47A, Japan).

Cell treatment

Murine melanoma cancer cell line (B16F10) was purchased from American Type Cell Collection. Normal human fibroblast (FHN) was gently donated by Professor Rui Curi (ICB-USP, SP, Brazil). B16F10 and FHN cells were routinely maintained in culture flasks (TPP, Switzerland), at 37°C in 5% CO_2, in DMEM with 100 IU/mL penicillin and 100 μg/mL streptomycin supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS, Invitrogen, USA). For most of experiments, B16F10 or FHN cells were seeded on 12-well plates at a density of 5×10^4 cells in DMEM containing 10% FBS (v/v) overnight at 37°C in 5% CO_2. The medium was changed and incubated without or with different concentrations of pentadactylin at 37°C in 5% CO_2. For the MTT assay of B16F10 and FHN cells, it was used a serial dilution of the peptide pentadactylin varying from 0 to 128 μM.

In order to investigate the mechanism of action of pentadactylin to B16F10 cells, a pentadactylin concentration of 30 μM was used. This value represents the minimal concentration capable to reduce the cell viability by 75% (IC_{75}), and it was calculated using the program Graph Pad Prism 5 (GraphPad Software). After 24 h, the cells were harvested by trypsinization, centrifuged, and prepared for the assays described below. All the experiments were realized in triplicate.

Cell viability assay

Cell viability was determined by a 3-4,5-dimethylthiazol-2,5 biphenyl tetrazolium bromide (MTT, Invitrogen, USA) assay (Mosmann 1983). Briefly, 15 μL of MTT solution (5 mg/mL in PBS) was added to each well. After 3 h of incubation at 37°C in 5% CO_2, the culture medium was aspirated and 100 μL of dimethyl sulfoxide was added. The absorbance was monitored using a spectrophotometer with a microplate reader at a wavelength of 595 nm (Bio-Rad, Hercules, CA).

Cell morphology analysis

Cell morphology of pentadactylin-treated (0, 32, 64, and 128 μM) B16F10 cells was analyzed using a contrast-phase microscope (Zeiss, Germany). The images were digitalized using a DCM35 camera connected to ScopePhoto© 1.0 (2003) computer software (Madell Technology Corporation, USA).

Plasma membrane integrity and cell count

B16F10 cells were mixed with trypan blue solution (0.4% in PBS, Sigma, USA) and counted by a hemocytometer

using a microscope. Cells with undamaged membrane were without dye staining inside and cells with disrupted membrane were with dye staining inside. The percentage of disrupted cell membrane was calculated based on the number of all counted cells.

Mitochondrial membrane potential

Cells were washed two times with 500 µL of PBS. Then, 0.5 µL of rhodamine 123 solution (5 mg/mL diluted in ethanol, Sigma, USA) was added to each cell group and incubated for 15 min at room temperature. The cells were washed two times with PBS and analyzed using a FACSCalibur flow cytometry (Becton, Dickinson and Company, USA). A total of 10,000 events were collected per sample. Rhodamine 123 is a cationic fluorescent probe shown to be selectively accumulated in mitochondria because of the transmembrane potential (inside negative) that these organelles maintain in living cells (Ronot et al. 1986).

DNA fragmentation and cell cycle analysis

Cells were ressuspended in 200 µL of 0.1% sodium citrate, 0.1% Triton X-100, 20 µg/mL propidium iodide, phosphate buffer solution (PBS), pH 7.4 (Invitrogen, USA) and incubated for 30 min at room temperature. The DNA fragmentation and the cell cycle were analyzed using a FACSCalibur flow cytometry (Becton, Dickinson and Company, USA). A total of 10,000 events were collected per sample. Propidium iodide is a fluorescent probe capable of binding and labeling DNA. Considering that propidium iodide fluorescence intensity is dependent on the DNA size, it is possible to precisely identify hypodiploid cells and cells on different cell cycle stages by flow cytometric analysis (Riccardi and Nicoletti 2006).

Statistical analysis

Statistical differences were evaluated using Student's *t* test, with significance $P < 0.05$. All values were expressed as mean ± SD. Each value is the mean of three different experiments in each group. The values significantly different from the control at $P < 0.05$ are indicated in the figures as asterisk.

Results

Purification of the AMP pentadactylin from the skin secretion of *L. labyrinthicus*

The chromatographic fractions obtained from the fractionation of the skin secretion of *L. labyrinthicus* by RP-HPLC procedure (Fig. 1a) were collected, and tested to their antimicrobial activity. One of the biological active fractions was submitted to further purification steps using a C_{18} column (Fig. 1b), being the resulted peptide analyzed using a Autoflex II TOF/TOF mass spectrometer in the reflection positive mode, obtaining its monoisotopic molecular mass $[M + H]^+$ of 2,541.29 Da (Fig. 1c).

The primary structure of the purified peptide was determined by automated Edman degradation, presenting the following sequence: ^{1}GLLDTLKGAAKNVVGSL ASKVMEKL^{25}. The mass difference observed between the experimental and theoretical molecular masses of the peptide indicates the presence of an amidated C terminus. It was confirmed by derivatization using Fisher esterification and mass spectrometric analysis of the modified peptide. The Fisher esterification is an acid-catalyzed reaction between alcohols and carboxylic acids to form esters and as a result of this reaction mass shift from the methylation of the accessible sites can be observed.

The amino acid sequence similarity search using NCBI non-redundant database did not reveal any similarity to other isolated peptides; on the other hand, the studied peptide was identified checking the available literature as being the peptide pentadactylin, an AMP isolated from the skin secretion of *L. pentadactylus* (King et al. 2005).

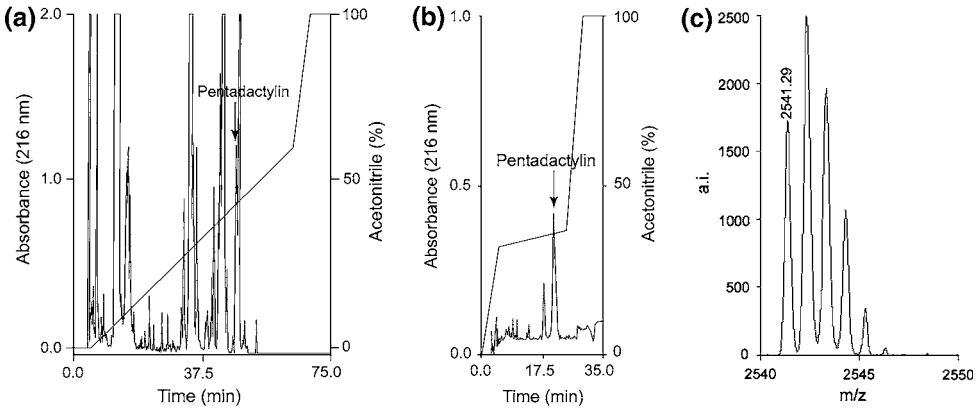

Fig. 1 **a** Chromatographic profile of crude *L. labyrinthicus* skin secretion (RP-HPLC; Vydac C_{18} 4.6 × 250 mm; flow rate = 1.0 mL/min). **b** Further purification of pentadactylin from *L. labyrinthicus* (RP-HPLC; Onyx monolithic C_{18} 4.6 × 100 mm; flow rate = 1.0 mL/min). **c** Mass spectrum of pentadactylin (Autoflex II TOF/TOF MS, Bruker Daltonics, Bremen, Germany, reflector positive ion mode)

Pentadactylin reduces cell viability of the murine melanoma cell line B16F10

Many researchers have shown that some cationic AMPs of broad spectrum are also cytotoxic to cancer cells and this stimulated our group to study the putative anticancer activity of pentadactylin. For the realization of the following assays a synthetic pentadactylin was utilized.

Pentadactylin reduced the cell viability of the murine melanoma cell line B16F10 after 24 h of treatment in a dose-dependent manner (Fig. 2). According to King et al. (2005), pentadactylin is not toxic to erythrocytes; however, to compare its cytotoxicity to other normal cell, we decided to test it against the normal human fibroblast cell line FHN. The results demonstrated that the peptide also reduced the cell viability of FHN cells (Fig. 2); nevertheless, it was more potent against B16F10. The IC_{50} values illustrate this difference: 35.9 μM to FHN and 25.7 μM to B16F10. Therefore, normal fibroblast cells are more resistant to pentadactylin treatment than B16F10 cancer cells. To elucidate the mechanism of action of this peptide, subsequent experiments were performed using the IC_{75} dose (approximately 30 μM), calculated using the program Graph Pad Prism 5.

Pentadactylin induces cell morphology alteration

Phase contrast micrographs showed that untreated tumor cells exhibited a normal morphology (Fig. 3a). The cells incubated with 32 μM (Fig. 3b) of peptide suffered alterations of nucleus morphology and loss of cell prolongations. Pentadactylin-treated cells exhibited complete loss of membrane integrity and acquired a round shape at 64 μM dose (Fig. 3c). At 128 μM, similar morphological features were observed, but at this concentration most of the cells acquired shrinkage morphology (Fig. 3d).

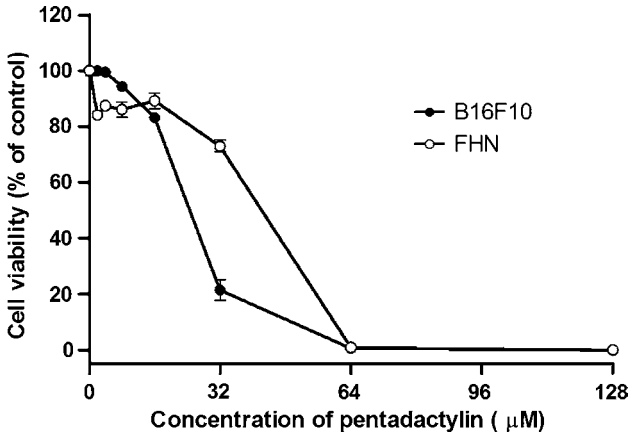

Fig. 2 Percentage of viable cells of the murine melanoma cell line B16F10 (*closed circle*), and the normal human fibroblast cell line FHN (*open circle*) after treatment with different concentrations of pentadactylin (2, 4, 8, 16, 32, 64, and 128 μM) for 24 h, and analyzed by MTT assay. B16F10 cells revealed to be more susceptive to pentadactylin than FHN cells. The values are expressed as mean ± SD of a triplicate experiment

The morphology of B16F10 cells treated with the IC_{75} dose was analyzed by flow cytometry to complement morphological data. In the group treated with pentadactylin, it was observed a 13.7% ($P < 0.01$) increase in the number of cells presenting altered morphology (left quadrants) compared to the non-treated cells (Fig. 4). These alterations corroborate the results obtained by microscopy.

Pentadactylin reduces the proliferation and damages plasma membrane of B16F10 cells

B16F10 cells incubated with 30 μM of pentadactylin were counted and their cell membrane integrity was analyzed. After 24 h of incubation, it was detected an anti-proliferative effect of the peptide. It induced a 60.7% decrease ($P < 0.01$) in the number of cells treated (Fig. 5a). Furthermore, 16.6% ($P < 0.01$) of the cells treated with the peptide showed plasma membrane disruption (Fig. 5b).

Pentadactylin induces alteration of the mitochondrial membrane potential, DNA fragmentation, and cell cycle arrest

To ascertain the cell death mechanism induced by pentadactylin, B16F10 cells were treated with 30 μM of peptide and flow cytometry analysis was performed. The data revealed a significant 10.3% increase ($P < 0.01$) of cells with altered mitochondrial membrane potential compared to the control (Fig. 6). It also showed 29.4% augment ($P < 0.05$) of DNA fragmentation after incubation with pentadactylin (Fig. 7). Pentadactylin-treated cells presented 16.4, 66.2, and 17.4% of G1 (Gap 1), S (Synthesis) and G2/M (Gap 2/mitosis) distribution of cells in the cell cycle phases, respectively. In contrast, non-treated B16F10 cells presented 56.9, 26.0, and 17.1% in the G1, S and G2/M phases, respectively (Fig. 8). Therefore, pentadactylin induced cell cycle arrest at S phase in B16F10 cells.

Discussion

It is the first time that the AMP pentadactylin is purified from the skin secretion of the pepper frog *L. labyrinthicus* (Fig. 1). Pentadactylin was first isolated from the skin secretion of *L. pentadactylus* by King et al. (2005). The presence of this peptide in two different species corroborates the results obtained by Hedges and Heinicke (2007). In their work the two species were part of the pentadactylus group according to mitochondrial DNA sequence analysis,

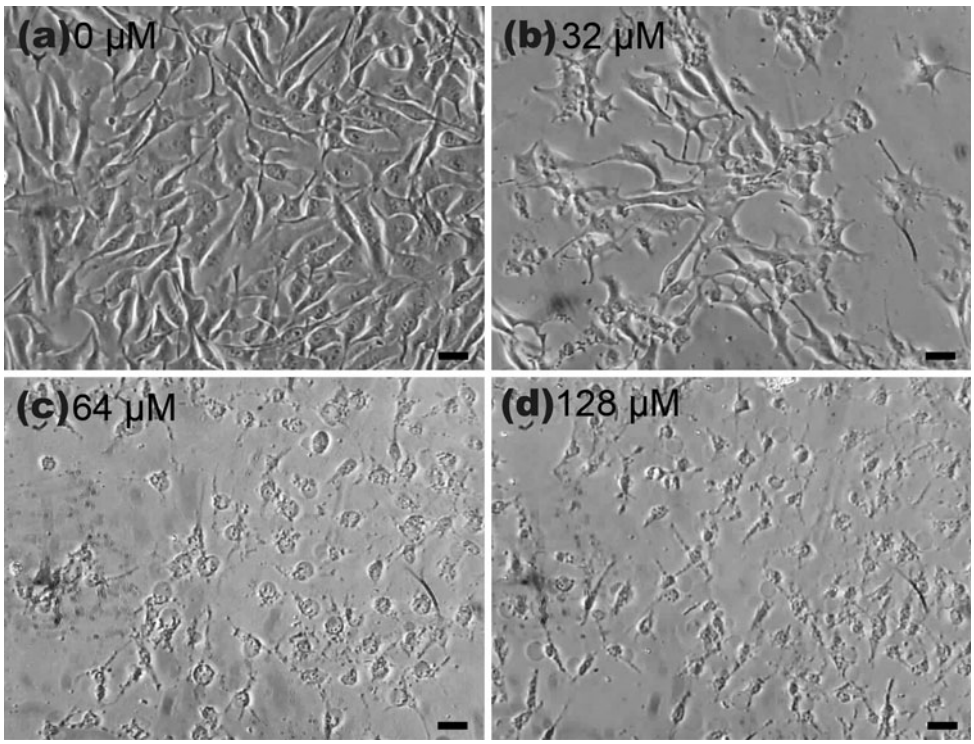

Fig. 3 Morphological alterations of B16F10 cells induced by different concentrations of pentadactylin observed by a contrast-phase microscope. **a** Control. **b** Cells treated with 32 μM presented a reduction of cell prolongations and loss of membrane integrity. **c** With 64 μM, B16F10 cells acquired a round shape and a complete loss of membrane integrity. **d** After incubation with 128 μM of pentadactylin the cells shrunk (*bar* 30 μm)

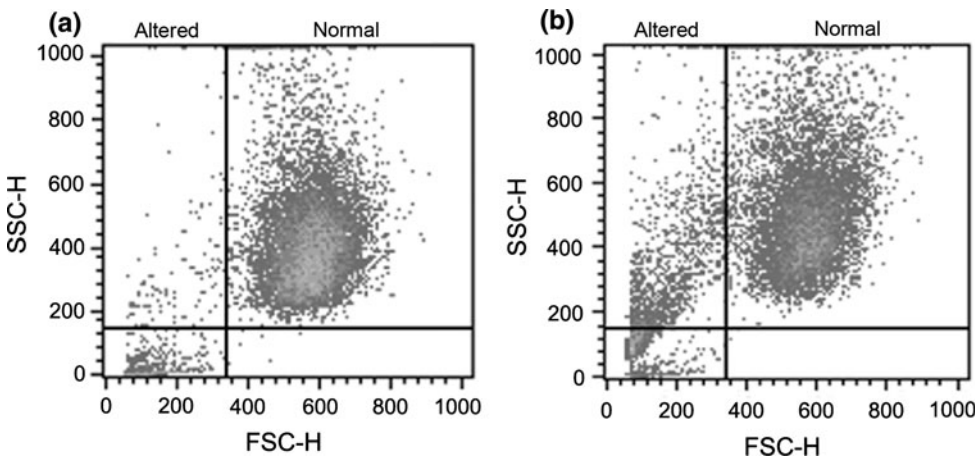

Fig. 4 Cell morphology analysis of B16F10 cells treated with 0 μM (**a**), and 30 μM (**b**) of pentadactylin for 24 h. Flow cytometry data show a 13.7% increase ($P < 0.01$) in the number of treated cells present in the left quadrants. These morphological alterations corroborate the results obtained by microscopy analysis

and they revealed to be the most related species among the ones analyzed. Nowadays, the use of distinct characters in evolutionary studies is common, resulting in more precise and trustworthy data. Thus, the study of peptides may also help to elucidate the history of the group Anura (Conlon et al. 2007).

Studies showing that some AMPs are also cytotoxic to cancer cells (Pukala et al. 2006) and the characteristics of pentadactylin lead us to investigate the hypothesis of an anticancer activity of this peptide. The MTT assay showed that pentadactylin reduced the viability of the murine cancer cell line B16F10 in a dose-dependent manner (Fig. 2). This result indicates that pentadactylin induced anti-proliferative and/or cytotoxic effects on these cells. Cell proliferation inhibition by an apoptogenic skin secretion compound of an anuran has been identified in the work developed by Gomes et al. (2007). Compared to the huge number of AMPs that have been isolated from amphibians, little research has been realized to verify the putative cytotoxicity of these peptides to cancer cells and their mechanism of action (Cruz-Chamorro et al. 2006).

Pentadactylin was also toxic to the normal human fibroblast cell line FHN. This result was not expected since pentadactylin did not show a significant cytotoxicity to normal erythrocytes in a previous study developed by King et al. (2005). Nevertheless, this discrepancy of results can be due to the fact that the activity of different cationic peptides is strongly influenced by the ionic strength

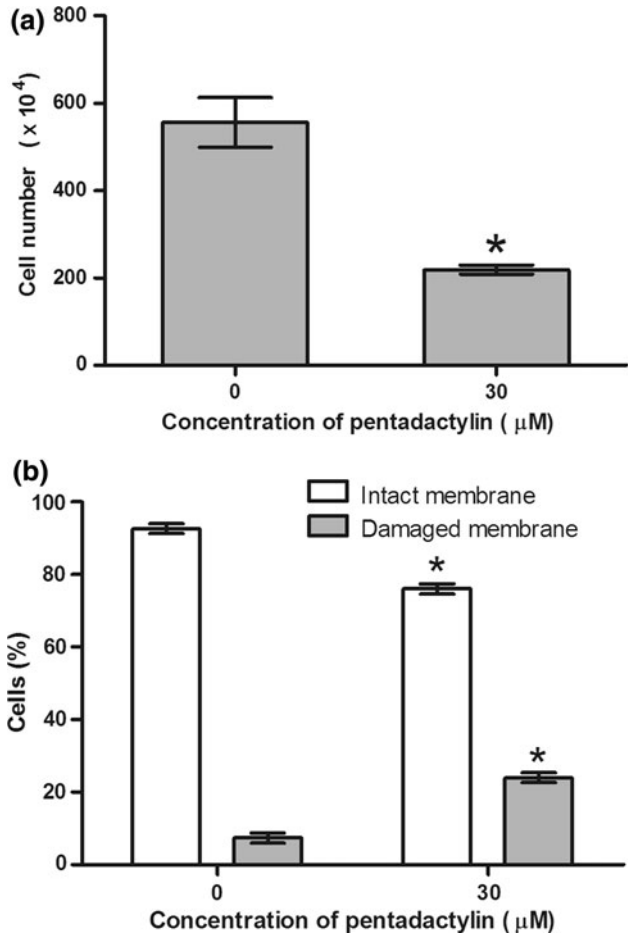

Fig. 5 Anti-proliferative effect of 30 μM pentadactylin to B16F10 cells. The results were obtained counting cells in the presence of trypan blue. **a** Pentadactylin induced a 60.7% decrease in cell number compared to the control. **b** The data revealed a 16.6% increase in the number of cells showing disrupted plasma membrane ($*P < 0.01$)

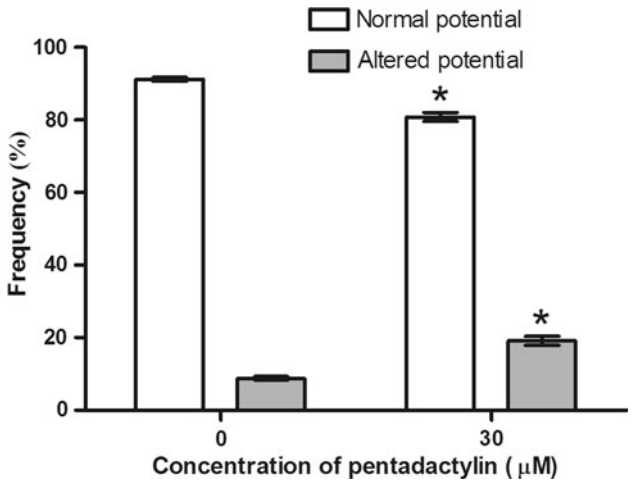

Fig. 6 Mitochondrial membrane potential of B16F10 cells after incubation with 30 μM pentadactylin for 24 h. The data revealed a significant 10.3% increase of cells with altered mitochondrial membrane potential compared to the control ($*P < 0.01$)

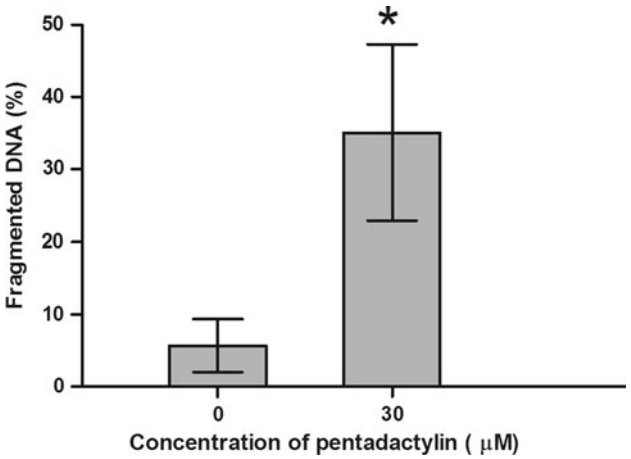

Fig. 7 DNA fragmentation of B16F10 cells treated with 30 μM of pentadactylin for 24 h. The percentage of DNA fragmentation was determined by quantifying cells showing DNA intensities less than the normal 2 N value. The results showed a 29.4% increase of fragmented DNA according to flow cytometry analysis ($*P < 0.05$)

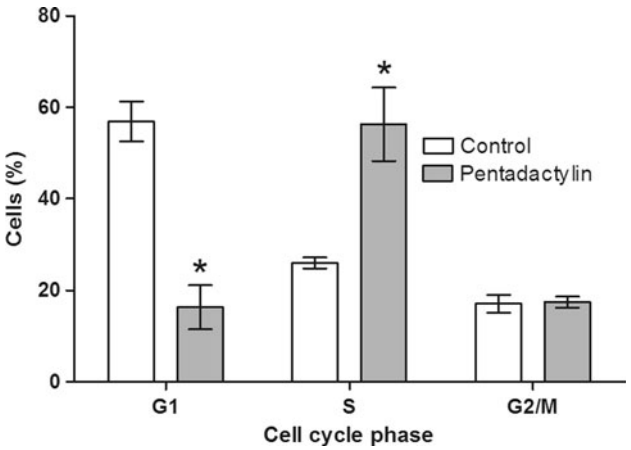

Fig. 8 Effect of 30 μM pentadactylin on the cell cycle phase distribution of B16F10 cells. The data show a cell cycle arrest with 40% increase of treated cells at the S phase compared to control. The results are presented as mean percentage cells in G1, S and G2/M ($*P < 0.01$)

(Helmerhorst et al. 1999). It was the case because the hemolytic assay was done in a saline buffer, which has a high ionic strength comparable to the medium used in the antitumor assay.

Despite the cytotoxicity to FHN cells, pentadactylin may be used as an alternative in melanoma therapy, since it presented a lower IC_{50} value to B16F10 cells than to FHN cells. Strategies that increase the selectiveness could reduce side effects, for instance, targeting the peptide to the tumor using carriers such as peptides that bind specifically to tumor cells (Enbäch and Laakkonen 2007), liposomes or the use of viruses (Guo et al. 2008).

Alterations on B16F10 cell viability were confirmed by phase contrast microscopy and flow cytometry. The

pentadactylin-treated cells presented modifications of cell morphology (Figs. 3, 4). In addition, the analysis using trypan blue confirmed the loss of membrane integrity of treated cells (Fig. 5b). According to Boleti et al. (2008), morphological alterations such as rounding and cell shrinkage are consistent with apoptotic cell death. These features were observed in their study with regard to the cytotoxicity of the lectin-like protein, pouterin, to tumorigenic and non-tumorigenic mammalian cell lines, concluding that pouterin induced apoptosis in the treated cells.

After treatment with the IC_{75} dose, the data showed a significant decrease in the total B16F10 cell number compared to the control, which suggested that pentadactylin interfered on B16F10 cell proliferation (Fig. 5a). Cell cycle study showed that pentadactylin (IC_{75}) induced a significant arrest of B16F10 cells at the S phase, and reduced the number of cells in G1 phase. According to Arellano and Moreno (1997) cell cycle progression is controlled by several Cyclin/CDK complexes. Furthermore, some studies have shown that besides inhibition of cyclin A/cdk2 kinase activity, p21 expression and inappropriate activation of E2F-1 may also be responsible for S phase arrest (Zhang et al. 2000; Zhu et al. 2004).

The investigation of the cell death mechanism revealed that pentadactylin-treated B16F10 cells had their mitochondrial membrane potential altered (Fig. 6) and DNA fragmented (Fig. 7). These data indicate apoptotic cell death and have been reported separately in the works of Tu et al. (2008) and Sun et al. (2002) about the apoptogenic mechanism of the honeybee venom and the box jellyfish toxin, respectively. It is known that genomic instability due to DNA fragmentation usually induces the activation of p53, resulting in growth arrest or apoptosis (Dasika et al. 1999; Igney and Krammer 2002). Besides, the DNA is the main target of a large variety of chemotherapeutics used in cancer therapy, acting in a direct or indirect way, or blocking metabolic functions involved with the DNA, for example, the DNA polymerase (Roos and Kaina 2006).

In the present study the rounding and cell shrinkage, DNA fragmentation, mitochondrial membrane potential alteration and S phase arrest support the apoptogenic nature of pentadactylin, whereas cell growth inhibition and reduction of cell viability indicate its anti-proliferative and cytotoxic activity. The exact mechanism that causes reduction of cell viability and cytotoxicity after pentadactylin treatment is still unknown. Apoptosis is an active and physiological mode of cell death subjected to complex regulatory processes. An important aspect of tumorigenesis, and of drug resistance development is the resistance to cell death. In this way, new strategies capable of inducing the apoptotic process in cancer cells are welcoming, modulating the sensitivity of tumor and normal cells to antitumor agents, via regulatory mechanisms of apoptosis (Okada and Mak 2004). Moreover, given that pentadactylin induces cell cycle arrest in B16F10 cells, it could potentiate the cytotoxicity of cell cycle-disrupting chemotherapeutics. There is, therefore, potential for the use of pentadactylin with cycle-dependent cancer drugs (Lam et al. 2009).

Besides the antimicrobial role of the cationic peptides purified from amphibians, many evidences indicate their use in cancer therapy, since AMPs present potent activity against tumor cells, are little immunogenic, and small, reducing their synthesis cost. The study about the mechanism of action, and the development of strategies capable of increase the potency, selectiveness, and resistance to serum components can represent an important progress of the therapeutic models (Cruz-Chamorro et al. 2006; Kim et al. 2003; Shadidi and Sioud 2003). Further studies are underway to evaluate pentadactylin as a complement to conventional cancer treatments. Nevertheless, it is not clear yet if anurans use the anticancer activity of AMPs, or if this activity is just a fortunate bonus originated from the membranolytic activity of these peptides (Doyle et al. 2003).

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