Evaluation of the antitumor and antiproliferative effects of the INKKI peptide on MCF-7 breast adenocarcinoma cells

Mariana Daou Verenhitach, Laertty Garcia de Sousa Cabral, Rosely Cabette Barbosa Alves and Durvanei Augusto Maria

Abstract
In this study we investigated the in vitro effect of the INKKI peptide in breast tumor cell lines and normal cells. The evaluation assessed the cytotoxicity activity effects, cell viability, mitochondrial electrical potential changing, effects on progression and cell cycle arrest and apoptosis markers involved. The results showed that the peptide INKKI features selective dose-dependent cytotoxicity in tumor cells, negatively modulating the mitochondrial electrical potential. The cell proliferation rate of the treated cells decreased, with cell cycle arrest in G0/G1. The peptide also able to induce apoptosis via mitochondrial pathway, which occurred independently of caspase 3 active. The INKKI peptide showed to be a potent modulator of antiproliferative and antitumor activities of the adenocarcinoma cell line MCF-7 human breast.

Keywords: INKKI, peptides, hydrolys, casein, breast cancer, caspase

1. Introduction
Breast cancer affects women in both developed and in developing countries, and is the leading cause of cancer death in women, accounting for 25.2% of all new cancer cases and 14.7% of deaths [1, 2]. In order to develop alternative therapies and which result in minimal side effects, naturally occurring peptides called bioactive peptides have been isolated from vegetal and animal sources in their original, synthetic or semi-synthetic forms, and tested in tumor cells [3, 4]. Casein is one of the most important and complex protein from bovine milk and compose approximately 80% of the total protein fraction of the milk. Casein is secreted almost exclusively in the form of large-dependent aggregates of calcium called micelles, and is composed of four subgroups of phosphoproteins, so-called polypeptide chains αs1 (119 amino acids), αs2 (207 amino acids), β (209 amino acids) and κ-casein (169 amino acids) [5]. The INKKI peptide corresponds to the fragment 41-45 of β-casein inserted into a β-pleated structure, and can be obtained by hydrolysis and amplified synthetically. The peptide has isoleucine residues at each end, giving it apolar character. Two lysine residues confer a positive charge, which together with asparagine residues confer hydrophobic character to the peptide [6]. We evaluated that INKKI peptide induced antitumor activity on murine melanoma B16-F10 cells without inducing detectable cytotoxicity in normal cells. The antitumor and antiproliferative effects are given by inducing apoptosis via caspase-3 and cell cycle arrest in increased DNA fragmented. In animal models inoculated with B16-F10 cells, the peptide decreased by 78.8% the tumor volume, induced necrosis and decreased the time of tumor growth and the metastasis formation rate [6]. The INKKI also showed to be a potent stimulator of phagocytic activity of macrophages treated with the peptide [7]. Our aim was to evaluate the antitumor and antiproliferative effects of the peptide INKKI on tumor cell lines of human breast adenocarcinoma MCF-7 is ER positive, PR positive, belongs to the luminal A molecular subtype. HER2 negative, and has low level of the protein ki-67, and the involvement of caspase-3 and 8 in the induction of apoptosis.

2. Materials and methods
2.1 The peptide
The synthetic peptide INIKKI used in this project was kindly provided by Dr. Ivo Lebrun of the Laboratory of Biochemistry, the Butantan Institute. The characterization peptide INKKI was described by Lebrun et al. [7].
2.2 Growing tumor cell lines and normal

The human breast cancer cell lines MCF7 (ATCC®™ HTB22), MDA-231 (ATCC® HTB-26™) and T47D (ATCC®™ HTB133); Ehrlich mouse breast cancer (ATCC® CCL-77™) and HUVEC human umbilical vein endothelial cells (ATCC® CRL 1730) were grown in RPMI 1640 medium (Sigma, St Louis, MO, USA). The culture medium were supplemented with 2 mM L-glutamine (CULTILAB, Campinas, SP, Brazil), 10 mM HEPES (CULTILAB, Campinas, SP, Brazil), 24 mM sodium bicarbonate, 0.1% of antibiotics and 10% fetal bovine serum (CULTILAB, Campinas, SP, Brazil), and were cultivated in 5% CO₂ atmosphere at 37°C in monolayers cultures. Cells were checked for viability by using the trypan blue exclusion test.

2.3 Determination of the cytotoxic activity

The cell viability assay was conducted to evaluate the cytotoxic effect of INKKI peptide and commercial chemotherapeutic drugs (positive controls paclitaxel and etoposide) at various concentrations in human breast tumor cells, murine tumor cells, endothelial normal cells and fibroblasts human dernal by the colorimetric MTT assay. The MTT [(3-(4,5-diethyl-thiazol-2-y1)-2,5-diphenyl tetrazolium bromide] (Sigma Chemical Co., St. Louis, MO, USA) colorimetric assay, is based on the reduction of formazan crystals by living cells. The cells were seeded in 96-well tissue culture plates at 2 × 10⁴ cells per well and incubated for 24h and then treated for 24h with different INKKI peptide concentrations diluted in RPMI-1640 culture medium at concentrations from 145 nM to 0.28 nM. Control cultures were treated with PBS. The plates were incubated at 37°C under 5% CO₂ for 24h. After treatment, the supernatants were removed, 100 µL of 5 mg/mL MTT solution was added to each well, and the plate was incubated for 3 h at 37°C with 5% CO₂. After this period, the medium was removed plus 100 µL of dimethyl sulfoxide (DMSO) (Sigma Chemical Co., St. Louis, MO, USA) to dissolve the formazan crystals formed and precipitated. The quantification of optical density was performed on ELISA reader (Multiskan-MS) at a wavelength of 540nm using the microplate reader Thermo Plate (Rayto Life and Analytical Science C. Ltd, Germany). The determination of the sensitivity of different doses has been optimized in accordance with the standards established by the National Cancer Institute, USA (National Cancer Institute). The IC₅₀ value, which represents the concentration of toxin needed to decrease viability to 50%, as compared to untreated cells, was calculated from the concentration-response curve.

2.4 Determination of the proliferative capacity by CSFE-DA

The evaluation of proliferative rate of tumor cells was performed by using the CFSE-DA (5(6)-carboxyfluorescein diacetate succinimidyl ester), intracellular fluorescent label which is divided equally between daughter cells. In this system, the cell division can be evaluated on multiple generations by flow cytometry, allowing for identification of up to 10 generations in vitro and in vivo. The cells were cultured for 96 h. The cell concentration of 5x10⁶ was incubated with CFSE-DA (50mM) for 20 min in an incubator at 5% CO₂ at 37°C, and after this period was proceeded a new counting. Subsequently, the cells were plated in wells at a concentration of 2 x 10⁵ cells diluted in 90 µL of RPMI with 5% FBS, with INKKI peptide in 10 L of RPMI with 5% FBS (basal), with chemotherapeutic agents (paclitaxel and etoposide) and with mitogens as positive controls (phytohemagglutinin and conconavalin-A). Finally, cells were incubated in CO₂ incubator at 37°C for 72h. After treatment, the cell suspensions were harvested, washed and fixed in 4% paraformaldehyde buffer. Analyses were performed using the percentage of responsive cells and the number of divisions that each cell analyzed suffered, discriminated according to the content of CFSE-DA by flow cytometry by FACSCalibur® (Becton-Dickinson Immunocytometry Systems, San Jose, CA, USA) and analyzed by CellQuest Pro program acquisition and analysis Modfit Becton-Dickinson (Modfit-BD).

2.5 Determination of the mitochondrial electrical potential by flow cytometry

Rhodamine 123 is a cationic lipophilic fluorescent chemical that accumulates specifically in mitochondria of living cells. For determination of mitochondrial electrical potential, cultures of MCF-7 cells were treated 24h with INKKI peptide, paclitaxel, etoposide. The cells were then incubated with rhodamine (Roh-123 - Molecular Probes®) for 30 min in a 5% CO₂ atmosphere at 37°C. The acquisition and analysis of the electric potential changes in the mitochondrial membrane were performed in the flow cytometer, and fluorescence intensity histograms with FL1-H parameters were acquired by the CellQuest Pro program.

2.6 The cell cycle phases

MCF-7 tumors cells (10⁴) were treated for 24h with INKKI peptide, paclitaxel and control with PBS alone. The cell suspensions were centrifuged twice at 3000 rpm for 10 min with PBS and resuspended in 200 µL solution of propidium iodide (20mg), 20 µL of Triton X-100 and 4 mg of RNase - A, for 30 min at room temperature and protected from light. After this period the samples were transferred to flow cytometry tubes. Images were acquired in the flow cytometer FACSCalibur® CellQuest Pro program, measured in the FL2 channel. The phases of the cell cycle pre and post-mitotic (hypodiplanoid, G0/G1, S and G2/M) were analyzed by Modfit-BD program.

2.7 Evaluation of Lipid Peroxidation

Supernatants from cell cultures used for the cytotoxicity assay were collected and frozen for the use in the assay of lipid peroxidation. The oxidative stress on unsaturated lipids in cell membranes was evaluated by determining the amount of malondialdehyde (MDA), which is the final product of fatty acid peroxidation that reacts with thiobarbituric acid (TBA) to form a colored complex, which can be measured in a spectrophotometer Multiskan MS-wavelength of 535 nm [8].

2.8 Evaluation of the expression of markers by flow cytometry

The profile of expression of markers involved in the cell cycle control and progression and in the mechanism of apoptosis was performed. The study measured the expression of the proteins p53, p21, p27, pRb, cyclin D1, the proapoptotic proteins Bad and Bax and antiapoptotic Bcl-2 protein, caspase 3 and 8, TRAIL-receptor DR4 (Abcam, Cambridge, MA, United States) and P-gp protein (Biogen, São Paulo, SP, Brazil), related to resistance against drugs. After 24 h, the treated and control cells were divided into cytometry tubes (10⁶ cells), being applied in each tube 10µL Triton-X100 for 30 min and then 1µg of each primary antibody. After 24h were applied secondary antibodies goat anti-human IgG-FITC
Flow cytometry was performed using FACSCalibur® cytometer and representative histograms were acquired by Cell-Quest Pro software with FL1-H parameters and FSC-H and analyzed with Modfit-BD program.

2.9 Statistical analysis
All values expressed the mean ± standard deviation. Each value is the mean of at least three independent experiments in each group. One-way analysis of variance (ANOVA) and Tukey-Kramer multiple-comparisons test was performed to identify differences Among measurements of the groups studied. Graphics were obtained by Prism version 5.0 and version 3.2 software ModFit. Statistical significance p-value *p <0.05, ** p <0.01 and *** p <0.001.

3. Results
3.1 Determining the cytotoxicity by MTT assay
The cytotoxicity of INKKI peptide in different concentrations was evaluated 24 h after treatment of tumor and normal cells by the MTT assay. The results demonstrated cytotoxicity in treatments using the INKKI peptide on tumor cells without toxic or inhibitory effects on the growth of normal cells. The Inhibitory Concentration IC50% value was 3.78 µg/mL to MCF-7 cells; 3.53 µg/mL for MDA-231; 3.26 µg/mL for T47-D and 3.07 µg/mL for Ehrlich tumor (Table 1). The concentrations shown on the graphs and figures which will be used later were determined by means of IC50% value, they are: INKKI peptide concentration of 3.78 µg/mL; paclitaxel chemotherapy concentration of 3.75 µg/mL and concentrations of etoposide to 4.81 µg/mL.

Table 1: Cytotoxicity activity of the INKKI on MCF-7 breast cancer cell line and normal cell.

<table>
<thead>
<tr>
<th>Cell Lineage - species</th>
<th>IC50%</th>
<th>R2</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-7 (human)</td>
<td>3.78 µg/mL</td>
<td>0.94</td>
</tr>
<tr>
<td>MDA-231 (human)</td>
<td>3.53 µg/mL</td>
<td>0.89</td>
</tr>
<tr>
<td>T47-D (human)</td>
<td>3.26 µg/mL</td>
<td>0.97</td>
</tr>
<tr>
<td>Ehrlich (murine)</td>
<td>3.07 µg/mL</td>
<td>0.95</td>
</tr>
<tr>
<td>Fibroblast FN-1 (human)</td>
<td>no effect</td>
<td>--</td>
</tr>
<tr>
<td>Endothelial CRL 1730 (human)</td>
<td>no effect</td>
<td>--</td>
</tr>
<tr>
<td>Control (MCF-7)</td>
<td>8.41 µg/mL</td>
<td>0.97</td>
</tr>
</tbody>
</table>

3.2 Analysis of the cell cycle phases
The INKK peptide induces in MCF-7 tumor cells changes in the percentage of cells in different phases of the cell cycle. Cells were treated with INKK peptide concentration in the concentration of 3.78 µg/mL for a period of 24 h. The results showed an inhibitory effect on the proliferation capacity (reduction of the population in phase G2/M) and increased the proportion of cells in sub-G1 phase. DNA fragmented the proportion of cells in G2/M phase of the group treated with the peptide was about 60% lower compared to the control group. Statistical correlations effectiveness and efficacy of treatment with INKKI peptide at a concentration of 3.78 µg/mL as compared to paclitaxel and representative histograms are shown in Figure 1 and clearly show the changes in decreased proportion of cells with proliferative potential inversely proportional to the fragmented DNA.

3.3 Determination of the mitochondrial electrical potential
The analysis of mitochondrial electrical potential (ΔΨm) was used the test of Rhodamine 123 in MCF-7 tumor cells were treated with INKKI peptide at a concentration of 3.78 µg/mL. Cells treated the INKKI peptide showed reduction of 36.4 ± 5.8% of mitochondrial activity, when compared with the control group. Paclitaxel chemotherapy was able to reduce an average of 93.2 ± 12.4% of mitochondrial potential (Figure 2).
Means and standard deviations of the mitochondrial potential of the INKKI peptide 3.78 μg/mL in MCF-7 cells by the Rhodamine 123 efflux method by flow cytometry after 24h of treatment with the peptide 3.78 μg/mL, paclitaxel 3.75 μg/mL and etoposide 4.81 μg/mL and the control groups. The bars in blue represent the population of cells with inactive potential, which do not present electric potential, and the bars in red the active cells, which present electric potential. B) Curves of the different electrical potentials of the mitochondria using the rhodamine 123 fluorescent probe by flow cytometry in MCF-7 breast adenocarcinoma cells treated 24h with the peptide INKKI 3.78 μg/L (green curve), paclitaxel 3.75 μg/mL (curve in red), etoposide 4.81 μg/mL (curve in orange) and control (curve in blue).

3.4 Determining the proliferative capacity
The results showed that the INKKI peptide at a concentration of 3.78 μg/mL, significantly inhibited about 28% of the proliferative response of breast adenocarcinoma MCF-7 tumor cells (Figure 3). Positive control cells were stimulated with phytohemagglutinin and / or concanavalin-A.

3.5 Determination of production lipid peroxidation
The quantification of lipid peroxidation of the tumor and normal cells was measured after 24 h of treatment with the peptide. Peroxidation levels are related in each line, the concentration of peptide INKKI. After the treatment period there was a significant increase in lipid peroxidation of MCF-7 tumor cells, reaching the highest percentage concentration of 7.2 μg/mL, obtaining lipid peroxidation value of 17.8 ± 0.8%, however, at the highest concentration tested (12.4 μg/mL) the percentage was 2.6 ± 0.3%. In HUVEC and FN1 normal cells, mean percentage values was smaller than the tumor cells, with values of 6.9% ± 0.2 and 9.8 ± 0.5%, respectively, at the highest concentration (Figure 4).
3.6 Cell markers expression by flow cytometry

After treatment of MCF-7 tumor cells with INKKI peptide at 3.78 µg/mL for a period of 24h, analysis of expression of proteins involved in apoptosis and proliferation control was performed. Treatment with peptide significantly decreased the expression of the protein cyclin D1, obtaining percentage of 37.7 ± 3.4%, compared to the control group with 87.2 ± 4.7%, important response inhibition proliferative.

The expression of the proteins in the check point process progressive of the cell cycle was also quantitated after the treatment period. It observed a significant increase in expression of the p53 protein (74.0 ± 4.4%), p21 (26.5 ± 4.3%), p27 (17.4 ± 2.6%), pRB (41.2 ± 2.0%) and P-gp (9.1 ± 1.5%) in the control group. Treatment with the peptide has altered the expression of apoptotic pathway and receptors of dead cells associated the expression of Bax protein (32.7 ± 4.9%), Bad (19.0 ± 1.5%) and DR4 receptor (19.8 ± 1.8%) increased significantly in the control group, the expression of anti-apoptotic protein Bcl-2 significantly reduced, getting value of 31.3 ± 2.5%, compared to control group. However, the expression of caspase 3 and 8 active were not changed significantly by INKKI peptide (Figure 5).
4. Discussion

Treatment of various cell types of human and murine breast cancer and normal cells showed that INKKI peptide has selective cytotoxicity for tumor cells, not resulting in significant changes in normal cells. The INKKI peptide was able to induce increased lipid peroxidation formation on tumor cells, dependent of concentration the treatment.

The main hypothesis for the selective cytotoxicity of amphipathic cationic peptides such as INKKI on tumor cells is due to the fact that the plasma membranes of tumor cells present higher electronegativity compared to normal cell membranes due to an increased expression of anionic elements such as heparan sulfate, phosphatidylserine and sialic acid, making tumor cells more susceptible to the uptake of amphipathic cationic peptides. Another feature of the tumor cells’ plasma membranes is the increased presence of microvilli, increasing the contact surface with this type of peptide [9, 10].

In addition to differences in the plasma membranes, the mitochondrial membranes of the tumor cells differ in relative to normal cells, and this stems from changes in the biochemistry of energy production, generating a higher mitochondrial electrical potential relative to normal cells. These changes may make the mitochondrial membranes more susceptible to amphipathic cationic peptides such as INKKI, which may act as mitochondria toxic agents acting directly on OM (outer membrane), penetrating it and generating pores [11-13]. In addition to the feature resulting from the differential electric potential, another factor that contributes to the mobilization of amphipathic cationic peptides to mitochondria is that the inner membrane (IM) presents more negatively charged than the OM due to differences in their lipid composition, i.e. presence of phospholipids acids, attracting amphipathic cationic peptides to the intermembrane space [13].

The formation of pores caused by the amphipathic cationic peptides action promotes the MOMP (mitochondrial outer membrane permeabilization), the main factor which triggers apoptosis. Once started, the process is irreversible and fatal [13]. Amphipathic cationic peptides were ranked by Galluzzi et al. as monotherapy agents, which can induce apoptosis via MOMP without the need for combined therapies. Among the prominent peptides with this capability are F16 molecule, peptide (KLAKLAK) 2 and mastoparan peptide. F16 peptide is a cationic amphoteric molecule directly involved in isolated mitochondrial membranes permeabilization [13]. In models of prostate tumors in mice, F16 was able to inhibit its growth [12]. The MOMP causes the permeabilization of the IM, depolarization of both mitochondrial membranes, accumulation of water inside the intermembrane space and inside the organelle, breaking the OM and consequently promoting the release of proapoptotic agents such cyclotchrome c, apoptosome and Smac/Diablo to the cytoplasm [14]. Cytotoxiccompounds effectors of caspase-independent death are also released, such as AIF and endonuclease G, which can act on the nucleus in an autonomous way [15].

In this study, the results showed that the INKKI peptide was capable of reducing the mitochondrial electrical potential in 36.4 ± 5.8% when compared to the control group, thereby demonstrating the ability of this peptide to alter mitochondrial metabolism, allowing induction of apoptosis. In addition to direct consequences of the NKKI penetration in the OM, the release of proapoptotic compounds of the mitochondria to the cytoplasm causes the activation of other agents who will act in an amplification loop on the permeabilization of mitochondrial membranes, reaching the mitochondrial permeability transition pore (PTP) [14].

MCF-7 tumor cells may exhibit deficient in the expression of active caspase-3 resulting from depletion of 47 base pairs in exon 3 CASP-3 gene, the gene of caspase 3 [16-18]. In this study, the results of the evaluation on the levels on the expression of caspase-3 and caspase-8 revealed no significant differences in the levels of expression of these proteins from control cells to treated cells, suggesting that apoptosis mechanism induced INKKI occurs in a caspase-independent manner. In other study, murine melanoma cells B16F10-Nex2 treated with mastoparan also showed no significant differences in the expression of caspase 8 of these cells compared the control group [19]. Although in theory the INKKI mechanism of action, as well as with other amphipathic cationic peptides on cell death, does not involve the extrinsic pathway of apoptosis, expression of caspase-8 was performed only to confirm the initial hypothesis that amphipathic cationic peptides induce cell death via mitochondrial pathway.

On this study there was increased expression of DR4 TRAIL receptor-receptor cells treated with INKKI compared to the control group, a fact related to the increased p53 expression, which is able to induce the expression of death receptors regardless of caspase expression [20, 21].

Under normal conditions, the p53 protein is present in basal and quiescent levels in the cells. DNA damage, cellular stress and hypoxia can promote the induction and upregulation of this protein [22]. Damage to DNA caused by AIF and endonuclease G induce the ATM activity and the release of p53 from its complex with Mdm2 [23] and induce increased p53 expression [24]. Treatment with INKKI peptide induced increasing of the p53 protein expression, which therefore can be positively regulating expression of proapoptoticproteins, thus being responsible for mediating induction of programmed cell death in MCF-7 tumor cells.

At the mitochondria, p53 can interact directly with the OM, increasing the MOMP, by itself corroborating with the release of the proapoptotic compounds to the cytoplasm [25]. The p53 protein can enlarge the induction of MOMP through a transcription-dependent activity, activating initially transcription of Puma and then ofBax, Bak, Noxa [13, 25-27] and Bad [26] and inhibitingthe transcription of anti-apoptotic proteins of the Bcl-2 family [24, 25]. In this study, we evaluated the levels of the expression of Bad, Bax and Bcl-2 in cells treated with the peptide INKKI. The INKKI peptide increased the expression of Bax and Bcl, reducingthe Bcl-2 expression. Thus, the data suggest that the regulation of these proteins after the treatment with the peptide are due to an increased expression of p53. In studies with murine melanoma B16F10-Nex2 cells treated with mastoparan, there was an increased expression of Bax, Bak and Bim and a decreased expression of Bcl-2 [19].

Alongside the proapoptotic effects of p53 upregulation induced by the peptide INKKI, p53 can act promoting an increased gene expression of cell cycle regulatory proteins such as p21 and GADD45 [23].The INKKI peptide at a concentration of 3.78 µg/mL significantly inhibited the proliferation of MCF-7 tumor cells, indicating the effectiveness of INKKI to promote cell cycle arrest. The cell cycle arrest in the G0/G1 phase can be explained by the results of markers related to cell cycle checkpoints analyzed by flow cytometry. Increased p21 expression was associated with increased p53 and p27 expression. In MCF-7 tumor cells treated with the peptide INKKI was observed cell cycle arrest in these specific.
stages and decreased expression of cyclin D1, a result consistent with the increased expression of p21 and p27 markers. The increasing in p21 and p27 is also responsible for the augmented levels of pRb ([39], which was observed in the results of flow cytometry. With the inhibition of the cyclin / CDKs complexes, the pRb protein is kept accumulated in a dephosphorylated form, kidnapping the transcription factors of the E2F family, a fact that is associated with G1 arrest ([29], 2013:

Tumor cells, regardless of its origin tissue, may exhibit overexpression of P-glycoprotein (P-gp), a transmembrane protein which can be related do chemoresistant tumors. Among the P-gp substrates are different types of drugs, including chemotherapy agents. P-gp identifies and expels them from the cell. Normally, the expression of P-gp is increased when the cell is exposed to drugs, but factors such as stress and drugs not identified as its substrate can also induce its increased expression ([30]. The mastoparan and their analogs are capable of activating the expression of P-gp ([31], 2013:

Our data demonstrated that INKKI peptides proved to positively modulate the P-gp expression, however, increased P-gp still didn’t prevent the continuity of the process of apoptosis.

5. Conclusions

The INKKI peptide is a potent modulator of antiproliferative and antitumor activities of the breast adenocarcinoma cell line, promoting programmed cell death by apoptosis via mitochondrial pathway in a caspase-independent manner.

Funding

Coordination of Improvement of Higher Level Personnel and Foundation for Research Support of the State of São Paulo CNPq process number 306124/2015-7 Maria DA.

6. References

27. Shamas-Din A, Brahamhata H, Leber B, Andrews DW, BH3-only proteins: Orchestrators of apoptosis,

