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An *in vitro* test of new peptide against melanoma

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Abstract

Peptides have fantastic functions like prions pathogens that cause transmissible diseases devoid of nucleic acid. Furthermore, because of their high specificity, peptides can be used in antitumor therapy to target specific targets. In this work we describe the *in vitro* antitumor action against B16F10 cells of a peptide named W12K obtained from immunoglobulin gene. As a result, we showed antitumor growing in experimental trials such as viability, migration, and cell cycle. In conclusion the present article describes a molecule from immunoglobulin with future potential for *in vivo* therapeutic test.

Keywords: Peptide; Antitumor Activity; Cell Cycle; B16F10 Melanoma; Aggressive Tumor Cells; Cell Migration

1. Introduction

In nature peptides have fantastic functions and capabilities, they build adaptable viral across species [1] and prions pathogens that cause a group of fatal neurodegenerative diseases by transmissible mechanism that are devoid of nucleic acid [2].

With the aim of exploring the antitumor capabilities of peptides as their high specificity and ability to inhibit important tumor pathways we test the potential of a molecule obtained by a group collaborator [3]. The important capacity of peptides to inhibit tumors has been previously demonstrated by our laboratory through various works [4-6]. The peptide W12K was obtained from locus immunoglobulin (Ig) gene heavy joining 2 (IGHJ2) with amino acid sequence SLGPWHPGHCL-NH2, amidated in C-terminal to increase peptide stability for potentials *in vivo* experiments. The selection of the sequence followed the criteria: presence of positively charged residues, net charge, isoelectric point, and alternation of hydrophobic/hydrophilic residues in the sequence, by using ExPASy Proteomics Tools Compute pI/MW and ProtParam. These selection criteria are hypothetically similar to protective molecules of natural immunity, [7] for example by phylogenetic evolution defensins are natural peptides with similarity in mammalian plants, bacteria and fungi [8]. Consequently, W12K is a genomic human. Immunoglobulin sequence with previous biologic action, such results justified the test against B16F10 murine melanoma tumor cells [3].

Finally, the peptide W12K had *in vitro* antitumor activity against B16F10. So these results support the hypothesis that genes encoding the peptide display a therapeutic activity earmarking it for future interest testing of antitumor in animal models.

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2. Material and methods

2.1. Cell cycle analysis with propidium iodide (PI)

Control cells were incubated with dimethylsulfoxid (DMSO) at a concentration of 20 mg/ml and treated cells were inoculated with 1 mM of peptide. Harvested tumor cells were washed twice with PBS by spinning at 300 g for 5 min and discarding the supernatant before resuspension of cells at 3 x 10^6 cells/mL in a cell suspension buffer (PBS + 2% fetal bovine serum, FBS; PBS + 0.1% bovine serum albumin, BSA). Cell suspensions in 500 µL buffer aliquoted in 15 mL V-bottomed polypropylene tubes received 5 ml of cold 70% ethanol dropwise with gently vortexing. Cells were fixed for at least 1 h at 4 °C prior to propidium iodide (PI) staining and flow cytometric analysis. Fixed cells were washed twice in PBS by centrifugation. One mL of PI staining solution at 50 µg/mL was added to the cell pellet. A final concentration 0.5 µg/mL in 50 µL of RNase A stock solution was also added to the cells and incubation was performed overnight (or at least 4 h) at 4 °C. Stored samples kept at 4 °C were 10^6 events analyzed by flow cytometry BD Accuri™ C6 Plus.

2.2. Cell lines and culture conditions

The murine melanoma cell line B16F10-Nex2 was originally obtained from the Ludwig Institute for Cancer Research (LICR), São Paulo branch. The cell line grew in RPMI-1640 (Gibco, Grand Island, NY) medium supplemented with 10 mM of 2-(4-(2-hydroxyethyl) piperazin-1-yl) ethane sulfonic acid (HEPES; Sigma-Aldrich, St. Louis, MO), 24 mM sodium bicarbonate, 40 mg/L gentamicin (Hipolabor, Minas Gerais, Brazil), pH 7.2, and 10% fetal bovine serum (Gibco, Grand Island, NY). Cells were cultured at 37 °C and 5% CO2 and 95% humidity in the atmosphere.

2.3. "MTT" formazan cell viability assay

In a 96-well plate, 10^3 B16F10-Nex2 cells were incubated with a serial dilution of W12K with 1 mM to 0.125 mM and 20 mg/ml DMSO+PBS as negative controls for 36 h. A 5 mg/mL stock solution of "MTT" (1-(4,5-Dimethylthiazol-2-yl)-3,5-diphenylformazan, Thiazolyl blue formazan) was prepared in sterile H2O and 10 µL was added to each well containing 100 µL culture medium. After 4 h, the MTT containing-medium was removed from each well with a pipette and 100 µL of 10% SDS in H2O was added to solubilize the formazan crystal. The solutions were analyzed in a SpectraMax M2e (Molecular Devices) measuring the absorbance at 550 nm with a 690 nm filter with values plotted and analyzed on GraphPad Prism software.

2.4. Selection and synthesis of peptide encoded by immunoglobulin gene

The peptide sequences used in the present work are: (WTFGQGTKVEIR-NH2) with amidated C-terminal, the peptide W12K was obtained from human immunoglobulin locus Kappa joining 1 (IGKJ1). Amino acid sequence WTFGQGTKVEIR-NH2. Hydrophobicity (0*00*0*+0.0+). Isoelectric point (pI) 8.56. Molar mass (M.M) 1393.7. Net charge +. The research exploiting the Gene database of the National Center for Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov). According to different criteria, i.e. presence of positively charged residues, net charge, isoelectric point, and alternation of hydrophobic/hydrophilic residues in the sequence, by using ExPASy Proteomics Tools Compute pI/MW and ProtParam (http://www.expasy.org/proteomics). Selected peptides were synthesised by solid phase peptide synthesis method using a multiple peptide synthesiser (SyroII, MultiSynTech GmbH), at CRIBI Biotechnology Center (University of Padua, Italy). The purity of peptides, evaluated by analytical reverse phase HPLC, was in the 80–90% range. The peptides were solubilised in dimethyl sulfoxide (DMSO) at a concentration of 20 mg/ml and subsequently diluted in sterile distilled water for experimental use. For all experiments, controls (in the absence of peptides) contained dimethyl sulfoxide at the proper concentration.

2.5. Wound-healing assay for cell migration

B16F10-Nex2 cells (3x10^5) were seeded in 12-well plates and allowed to adhere and grow to 70-80% confluence. Peptides were added at 1 mM on adherent cells an cells control with (DMSO) at a concentration of 20 mg/ml; and further incubated for 12 h before scratching the cell monolayer with a sterile 1,000-µL-micropipette tip on each well. Cellular debris were removed after scratching and smoothing the edge of the scratch was achieved by washing the cells once with 1 ml of the culture medium and then replacing with 5 mL culture medium containing the peptides for the *in vitro* wound healing assay.

For image acquisition, the distances were calibrated with a micrometer blade; tip marks bordering the scratch were made on the outer bottom of the dish. Finally, the dish is examined in a phase-contrast microscope, leaving the reference mark outside the capture image field. Images were taken at 12 h regular intervals up to 36 h and the distance of cell

migration was measured using ImageJ software. By comparing the images from time 0 with those at other time points, we quantified the distance of each scratch closure determined by the cell front and measured as indicated above.

3. Results and discussion

Firstly, to analyze the direct effect of W12K on cell death [1] was used Trypan blue exclusion staining, as soon W12K peptide have a toxic effect only at doses superior at 1.5 mM (data no shown), the max 1 mM dose used not cause unspecific death so is safe to answer the experimental questions. Next, MTT assay showed that W12K treatments (0,25 mM, 0.5 mM, and 1 mM) decreased melanoma cell viability compared to controls after 36h (Figure. 1) thus this result indicates that the W12K acts on cell viability.

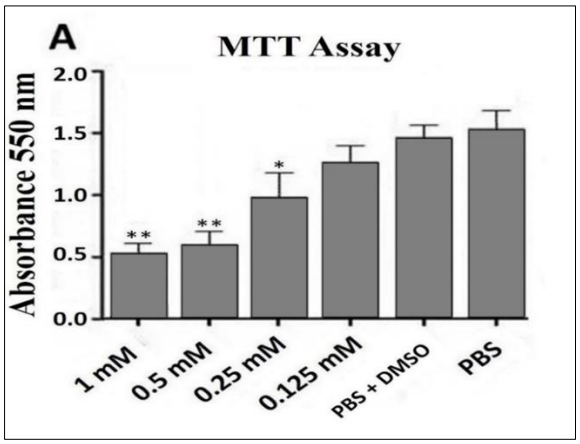

Figure 1 Viability of B16F10 cells treated with W12K

Absorbance reading at a wavelength of 550 nanometers (nm) for B16F10 cells cultured in the presence of W12K in serial dilutions of 1 mM, 0.5 mM, 0.25 mM, and 0.125 mM for 36 hours. The MTT viability assay showed a statistically significant difference between W12K treated cells at 1 mM, 0.5 mM and 0.25 mM versus controls PBS and PBS + DMSO. The graphs were made using the GraphPad Prism program using Bonferroni's Multiple Comparison and T-test. (* = $p < 0.05$) and (** $p < 0.01$).

The wound-healing assay was used to answer the question, if the W12K peptide has inhibition in tumor cell migration. Thus, the treatment at 1 mM, significantly reduced cell migration capacity, after 36 h incubation the cell wound was unfilled as seen on (Figure. 2 A-B); in contrast after 36 h, PBS control cells migrated to completely and fill the wound gap (Figure. 2 A-B), and control PBS+DMSO had a residual effect due to DMSO (Figure. 2 A-B).

(A-B) The migration ability of tumor cells was determined using the wound-healing assay. The W12K was added at 1 mM and images were taken at regular intervals of 12 h; the graphics represents the area between cells. Bar is 150 µm. Graph of area, black PBS control, gray treated W12K and light gray PBS + DMSO control. The area of cell migration was measured in µm by ImageJ. (A-B) Using peptide W12K, with delayed migration after 24 h, the wound was still open after 36 h; (B) Using PBS as control, the wound was completely healed after 36 h; (B) Using PBS + DMSO, the wound was practically healed after 36 h. (Figure 2).

As last experiment we used flow cytometry to analyze the cell cycle using propidium iodide DNA staining. We observed a difference between W12K and control PBS+DMSO treated cells, where was verified an increase of G1 phases and consequently decrease in the G2M phase (Figure. 3 A and B).Lastly, the effect on cell cycle could be explained by the interaction of W12K with cycle molecules, thereby the tumor cells in W12K treatment are unable to precisely make the check point between the G1 and S phases and as a consequence of this, there was an expansion of the S phase and a shrinkage of G2/M phases observed in (Figure. 3 A-B) [9, 10]; in the future this result can be elucidated by Western blotting of the molecules involved in the cell cycle.

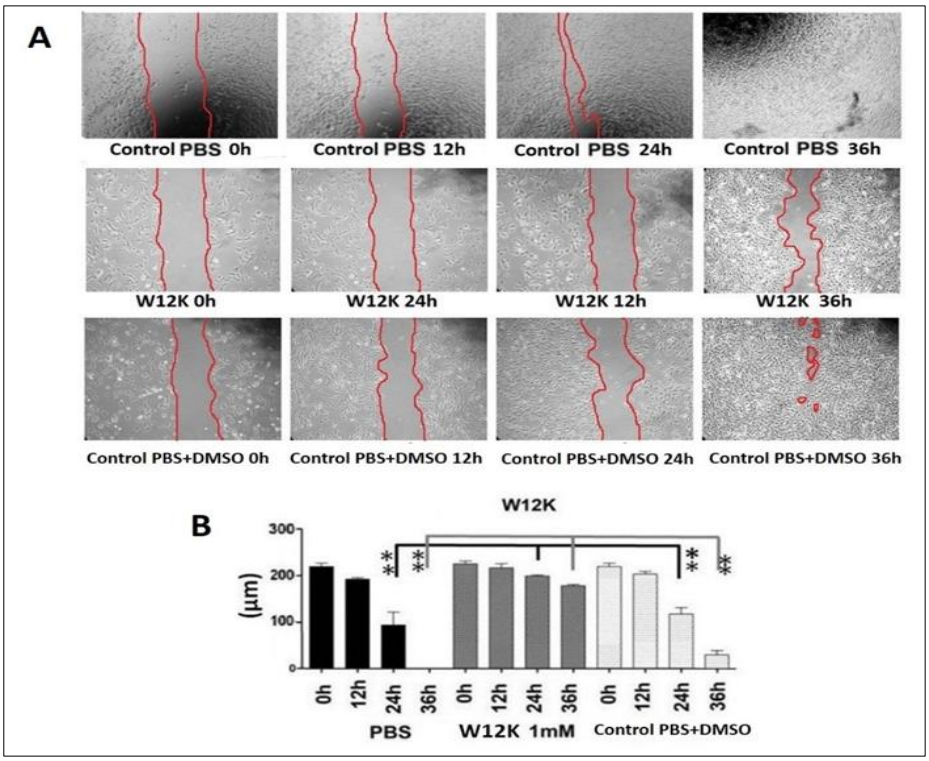

Figure 2 Wound-healing area after W12K treatment

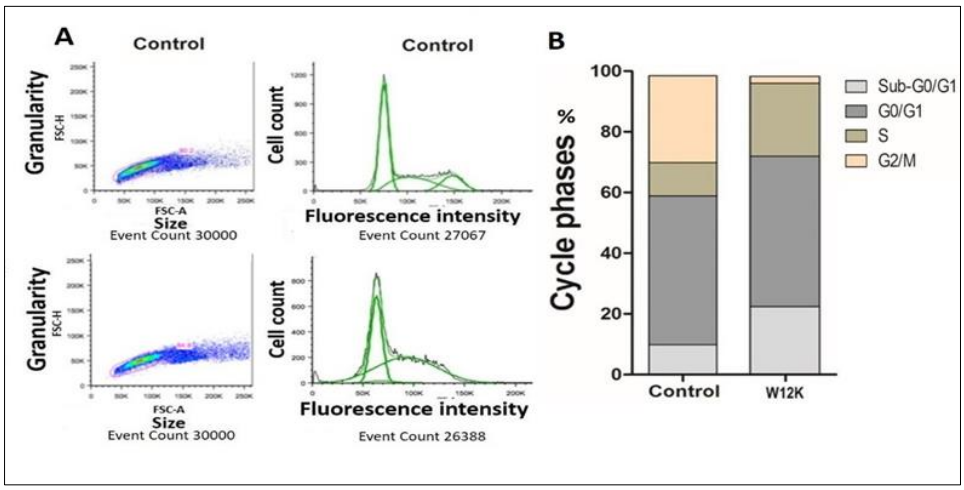

Figure 3 Cell cycle pattern by W12K treatment

10^6 B16F10 cells were treated with W12K and 3 x 10^4 analyzed by flow cytometer after incubation with propidium iodide (PI) solution. (A) PI staining shows different intensities in the population of W12K treated cells with difference in cell cycle pattern in comparison to PBS + DMSO control. (B) Percentage of each phase of the cycle in G1, S and G2 phases. The graphs and analyzes were made using FlowJo software.

4. Conclusion

Finally, this study will benefit the society to obtaining peptide sequences that have therapeutic action against melanoma and possibly for many other types of cancer. Thus we homologate the peptide for future in vivo assays. The W12K acts on cell viability, by inhibition in tumor cell migration and disturbing the cell cycle by an increasing in G1 and sub G1/G0

and S phase with great decreasing of G2M phase, and because that causing a delay in the cell cycle, fact that confirms the data in MTT and cell migration.

Compliance with ethical standards

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Consent for Publication

We authorize the full disclosure of the manuscript text and data.

Disclosure of conflict of interest

The authors declare that there is no conflict of interest.

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