

(RESEARCH ARTICLE)



L18R a peptide with potential against melanoma

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Abstract

Peptides have fantastic functions like defensins that make up the natural defense in different species from vertebrates to bacteria. Another fantastic example are prions that cause transmissible diseases devoid of nucleic acid. So, because of their high specificity, peptides can be used for different therapeutic goals. In this work we describe *in vitro* action of a peptide named L18R this sequence is part of a group of four distinct sequences that were previously tested and showed activity against fungi and tumors, which motivated us to investigate this activity against murine B16F10 melanoma. Thus, has been demonstrated that L18R had activity against tumor, for interfering in cell viability, migration and in cell cycle. To conclude, the present work suggests a functional molecule against tumor.

Keywords: Peptide; Antitumor activity; Cell cycle; B16F10 melanoma; Aggressive tumor cells; Cell migration

1. Introduction

Peptides have incredible capabilities and importance for agents such as viruses and their fantastic adaptations between species and terrible pandemic threats. For example, prions cause various fatal neurodegenerative diseases by a transmissible mechanism without nucleic acid, in addition there are also novels neurotropic viral strains capable of causing prion diseases [1, 2] so we can understand how dynamic the protein world is.

In this preliminary work, we evaluated the *in vitro* potential for therapeutic exploitation of an immunoglobulin (Ig) peptide amidated in C-terminal to increase peptide stability for future *in vivo* test [3, 4].

Following the same criteria of previously reported sequences [5]: The L18R was obtained by 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. So, through these criteria we can obtain molecules similar to protective molecules of natural immunity, which by phylogenetic evolution analysis look like defensins, [6,7]. Consequently, L18R is a genomic human immunoglobulin sequence with previous biologic action [3-5], such results justified the test against B16F10 murine melanoma tumor cells.

Finally, we show that peptide L18R had *in vitro* antitumor activity against B16F10, demonstrating the hypothesis that our selection criterion is capable of generating molecules that display therapeutical activity, for testing of antitumor activity *in vivo*.

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2. Material and methods

2.1. Cell adherence assay

Viable cells were seeded (1×10^6) in a 6-well plate and treated with 1 mM peptides for 36 h. The cells were detached with trypsin (0.025%) in PBS-EDTA (0.5 mM) for five minutes at 37 °C, thus obtaining loose cells without mechanical action. Trypan blue. The number of cells in 10 μ L transferred to a Neubauer chamber was counted with the aid of a manual counter. Cell adhesion was determined by the following formula: mean of four fields, multiplied by 1×10^4 as a correction factor and by 2 as a dilution factor, equivalent to the number of cells per mL. Adhesion (%) was compared to shedding of the treated cells versus the respective SCR and the untreated control system. The procedure was repeated 4 times and the graphs were performed using the GraphPad Prism program.

2.2. Cell cycle analysis with propidium iodide (PI)

Control cells were incubated with dimethylsulfoxid (DMSO) and treated cells were inoculated with 1 mM of peptide L18R. 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 3×10^6 cells/mL in a cell suspension buffer (PBS + 2% fetal bovine serum, FBS; PBS + 0.1% bovine serum albumin, BSA). Cell suspensions in 500 μ L buffer were aliquoted in 15 mL V-bottomed polypropylene tubes received 5 ml of cold 70% ethanol, dropwise with gently vortexing. Cells were fixed in ethanol 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 as described above. One mL of PI staining solution at 50 μ g/mL was added to the cell pellet. A final concentration 0.5 μ g/mL in 50 μ L of RNase A stock solution was also added to the cells and incubation was performed overnight (or at least 4 h) at 4 °C. 10^6 events of stored samples kept at 4 °C were analyzed by flow cytometry using the BD Accuri™ C6 Plus and the FlowJo software.

2.3. 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) ethanesulfonic 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.

2.4. “MTT” formazan cell viability assay

In a 96-well plate, 10^3 B16F10-Nex2 cells were incubated with a serial dilution of L18R, from 1 mM to 0.125 mM, and 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 μ L was added to each well containing 100 μ L culture medium. After 4 h, the MTT containing-medium was removed from each well with a pipette and 100 μ L 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.

2.5. Selection and synthesis of peptide encoded by immunoglobulin gene

The peptide sequences used in the present work is SLGPWHPGHCL-NH₂, with an amidated C-terminal, called peptide L18R. This sequence was obtained from human immunoglobulin locus heavy joining 2 (IGHJ2). Hydrophobicity (0000+*0000+00+*00+). Isoelectric point (pI) 10.35. Molar mass (M.M) 2068.1. Net charge 4+. The research exploiting the Gene database of the National Center for Biotechnology Information (NCBI, <https://www.ncbi.nlm.nih.gov>). 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 dimethylsulfoxide (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 dimethylsulfoxide at the proper vehicle concentration.

2.6. Wound-healing assay for cell migration

B16F10-Nex2 cells (3×10^5) were seeded in 12-well plates and allowed to adhere and replicate to 70-80% confluence. Peptides were added at 1 mM on adherent cells and further incubated for 12 h before scratching the cell monolayer with a sterile 1,000- μ L-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. Cells incubated with DMSO at a concentration of 20 mg/ml were used as control.

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

At first, it was necessary to change the sequence original from L18R molecule [3,4] because of difficult solubility in the melanoma culture medium, five terminal amino acids and two C-terminals were removed thus improving solubility and decreasing peptide toxicity.

Figure 1 *In vitro* cell adherence assay

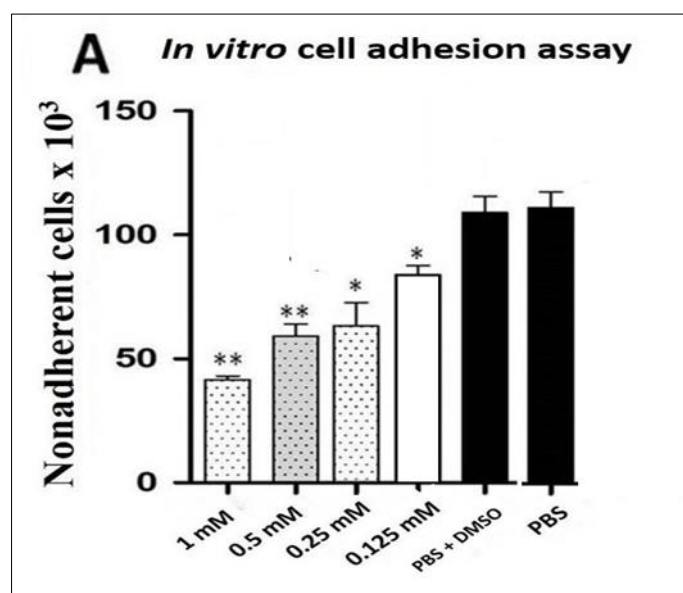


Figure 1 *In vitro* cell adherence assay. (A) The number of free cells was higher in controls PBS+ DMSO and PBD (black bars) compared to B16F10 cells cultured in the presence of L18R with serial dilutions of 1 mM, 0.5 mM, 0.25 mM and 0.125 mM for 36 hours (gray bars). The graphs were made using the GraphPad Prism software and using Bonferroni's Multiple Comparison and T-test as a statistical analysis. (* = $p < 0.05$) and (** $p < 0.01$).

Trypan blue exclusion staining was used to analyze the live and dead cells. Since L18R peptide only presented any toxic effect at concentrations superior of 1.5 mM (data no shown), the maximal of 1 mM doses were used in the experiments reported, avoiding any unspecific death and being safer to answer the experimental questions.

To verify the direct effect of L18R peptide on *in vitro* cell adhesion, we have used a cell adherence assay that showed an increased adhesion rate in melanoma cells as compared to control (Figure. 1). So, the treatment with L18R probably increased the expression levels of adhesion protein E-cadherin and decrease in N-cadherin in cells (Figure. 1), a sign of decreased tumor cell aggressiveness, since the increased expression of E-cadherins keeps the tumor cells fixed to the source organ and decrease metastasis but N-cadherin allows fixation in other organs contributing to increased metastases [8,9]. So, to answer the question how L18R effects cell adhesion, the L18R peptide could reverse a cadherin adhesion aggressive phenotype based on this result. However these data need to be confirmed through Western blotting (WB)

The MTT assay showed that L18R treatments (0,25 mM, 0.5 mM, and 1 mM) decreased melanoma cell viability compared to controls after 36h (Figure. 1). This result indicates that the L18R acts on cell viability, thus being possible to also test cell proliferation in future studies.

Figure 2 Viability of B16F10 cells treated with L18R

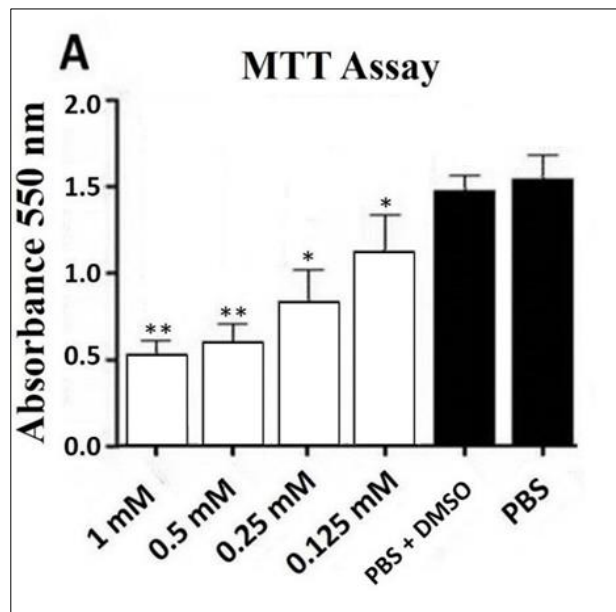


Figure 2 Viability of B16F10 cells treated with L18R. (A) Absorbance reading at a wavelength of 550 nanometers (nm) of B16F10 cells cultured in the presence of L18R with serial dilutions of 1 mM, 0.5 mM, 0.25 mM and 0.125 mM for 36 hours. The MTT viability assay showed a statistically significant difference between L18R 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 software and using Bonferroni's Multiple Comparison and T-test as a statistical analysis. (* = $p < 0.05$) and (** $p < 0.01$).

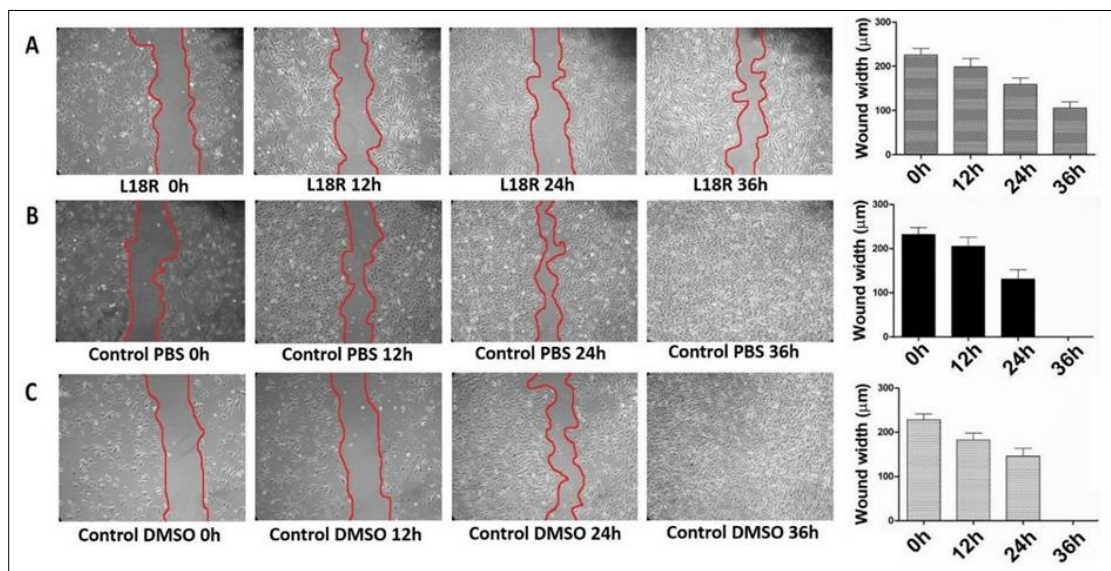


Figure 3 Wound-healing assay after L18R treatment shows impaired cell migration. The migration ability of tumor cells was determined using the wound-healing assay. The L18R peptide was added at 1 mM concentration and images were taken at regular intervals of 12 h; the graphics represents the area between cells. Graph of area, black PBS control, gray treated L18R and light gray PBS + DMSO control are shown on right panels. The area of cell migration was measured in μm using the ImageJ software. (A) Using peptide L18R the wound was still open after 36 h, with clear delayed migration after 24 h; (B) Using PBS as control, the wound was completely healed after 36 h; (C) Using PBS + DMSO, the wound was completely healed after 36 h.

The wound-healing assay was used to answer the question if L18R peptide can inhibit tumor cell migration. Thus, the presence of L18R in middle at 1 mM significantly reduced cell migration capacity. After 36 h of incubation, the L18R-treated cells wound was still unfilled, as seen on (Figure. 3A). In contrast, after 36 h, control PBS –treated cells migrated

to completely fill the wound gap (Figure. 3 B), and also control PBS+DMSO-treated cells behaved like the PBS-treated cells, with normal cell migration to fill the wound (Figure. 3 C). This way, we detect a direct effect of the peptide L18R on cell migration, aiming for a future clonogenic assay to observe the toxic effect of the peptide.

As a last experiment on this report, we used flow cytometry to analyze the cell cycle of melanoma cells stained with propidium iodide. We observed a difference between L18R and control PBS+DMSO treated cells, where it was verified an increase of G1 and S phases, consequently with a decrease in the G2M phase, on L18R-treated cells (Figure. 4 A and B).

Figure 3 Wound-healing assay after L18R treatment.

Finally, the alteration on cycle can be theorized by interaction of L18R with cycle molecules, preventing tumor cells from performing an adequate check point between the G1 and S phases, thus causing an effect of expansion of the S phase and a shrinkage of G2/M phases observed in (Figure. 4 A-B) [10, 11]; so, we intend to clarify the data with new experiments by Western blotting of the cell cycle signaling paths.

Figure 4 Cell cycle modulation with L18R treatment.

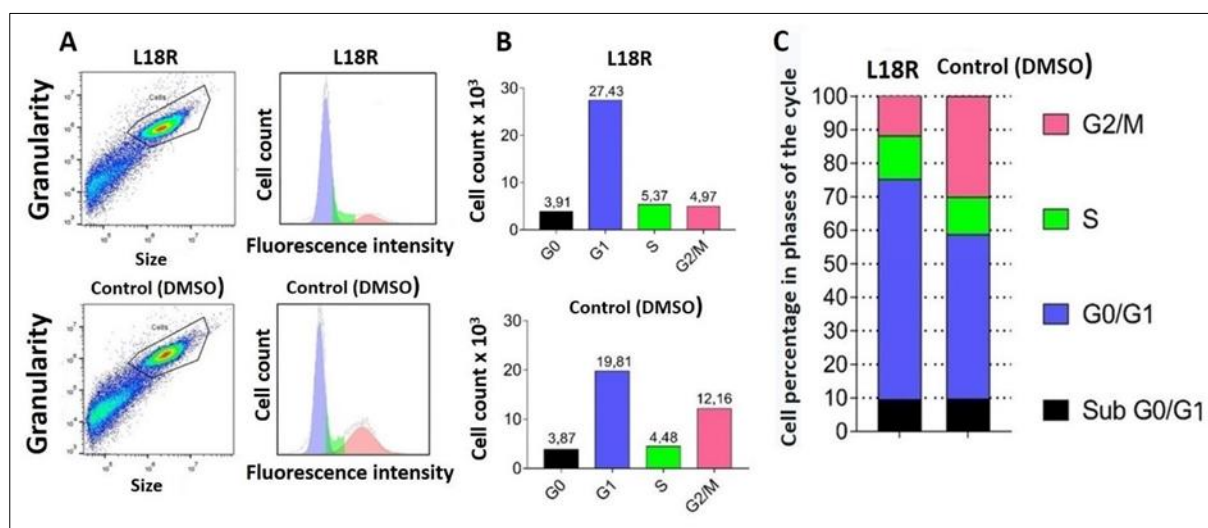


Figure 4 Cell cycle modulation with L18R treatment. 10⁶ B16F10 cells are treated with L18R and analyzed by flow cytometer after incubation with propidium iodide (PI) solution. (A) PI staining shows different intensities in the population of L18R treated cells with difference in cell cycle pattern in comparison to PBS + DMSO control. (B) Number of cells in each phase of the cycle showed L18R treated and control PBS + DMSO in G1, S and G2 cell cycle phases. (C) Percentage of each phase of the cycle in G1, S and G2 phases. The graphs and analyzes were made using FlowJo and GraphPad software.

4. Conclusion

The L18R acts on cell adherence, viability and cycle inhibiting tumor cell migration and disturbing normal cycle. The increase in G1 and S phase, with decreasing G2M phase, are typically caused by the delay in the cell cycle, which can explain the observed data in MTT and cell migration of L18R-treated melanoma cells.

Finally, we show that peptide L18R had *in vitro* antitumor activity against B16F10, demonstrating the hypothesis that our selection criterion is capable of mimic molecules similar to protective molecules of natural immunity, to obtain and generating molecules that display biological activity, against melanoma *in vitro* and encouraging the test in many other types of cancer *in vitro* and *in vivo*.

Compliance with ethical standards

Acknowledgments

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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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