World Journal of Advanced Pharmaceutical and Life Sciences

Journal homepage: https://zealjournals.com/wjapls/

ISSN: 2799-0222 (Online)

(RESEARCH ARTICLE)

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Immunoglobulin peptide against melanoma

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World Journal of Advanced Pharmaceutical and Life Sciences, 2022, 02(01), 009–013

Publication history: Received on 14 December 2021; revised on 13 January 2022; accepted on 15 January 2022

Article DOI: https://doi.org/10.53346/wjapls.2022.2.1.0037

Abstract

Was investigated a peptide named L12P obtained from human immunoglobulin Lambda joining 1 (IGLJ1*)* before recombination. The peptide previously showed activity against fungi, which motivated us to investigate the antitumoral activity against murine B16F10 melanoma tumor cells. To test the biological activity of the peptide against tumor cells different tumor phenotypes were initially assayed *in vitro*, such as migration, adherence and cell cycle. Hence, the selected peptide displayed significant action *in vitro* against melanoma cells. In conclusion the present work may suggest a functional molecules derived from immunoglobulin with future potential therapeutic.

Keywords: Peptide; Antitumor activity; Cell cycle; B16F10 melanoma; Cell migration

1. Introduction

In this preliminary work we confirm the potential for therapeutic exploitation of a peptide obtained by a group of Italian collaborators [1]. Your data have demonstrated the efficiency of several peptide sequences against fungi, and our laboratory through collaboration tests the efficiency of these peptides against tumors [2].

Considering old gene segments before recombination is hypothesized, a role similar to the protective molecules of natural immunity, raising a question about their phylogenetic evolution, [3] for example, defensins are natural peptides in the mammalian immune system that share molecular similarity with plants, bacteria and fungi [4]. Consequently, L12P is a genomic human immunoglobulin (Ig) sequence with previous action against fungi; such results justified the test against B16F10 murine melanoma tumor cells [1,2].

Finally, the peptide L12P had *in vitro* antitumor activity against B16F10. These results support the hypothesis that genes encoding peptides display a therapeutic activity which includes future antitumoral test in animal models.

2. Material and methods

2.1. Cell cycle analysis with propidium iodide (PI)

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 + 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)

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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) 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% CO_2 and 95% humidity in the atmosphere.

2.3. MTT cell viability assay

In a 96-well plate, 10^3 B16F10-Nex2 cells were incubated with a serial dilution of L12P and SCR peptides and PBS as negative controls for 36 h. A 5 mg/mL stock solution of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was prepared in sterile H_2O 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 H_2O 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 L12P was obtained from Locus Lambda Gene of Immunoglobulin Junction Light 1 (IGLJ1). Amino acid sequence LCLRNWDQGHRP. Hydrophobicity (0*0+*0-*0++0). isoelectric point (pI) 8.26. molar mass (M.M) 1494.7. Net charge 2+. 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 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 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 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

After 36h MTT assay showed that L12P treatments, decreased melanoma cell viability compared to controls PBS and PBS+DMSO, with statistical difference in following concentrations (0,25 mM*, 0.5 mM**, and 1 mM**) by Bonferroni's Multiple Comparison and T-test. (* = $p < 0.05$) and (** $p < 0.01$) see (Figure. 1) this result indicates that the L12P acts on cell viability.

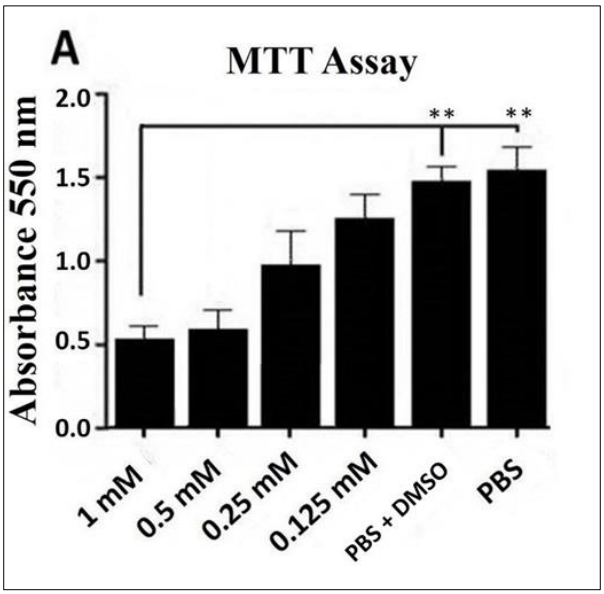

Figure 1 Viability of B16F10 cells treated with L12P. Absorbance reading at a wavelength of 550 nanometers (nm) for B16F10 cells cultured in the presence of L12P in serial dilutions of 1 mM, 0.5 mM, 0.25 mM, 0.125 mM and 0.065 mM for 36 hours. The MTT viability assay showed a statistically significant difference between L12P 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 (** $p < 0.01$)

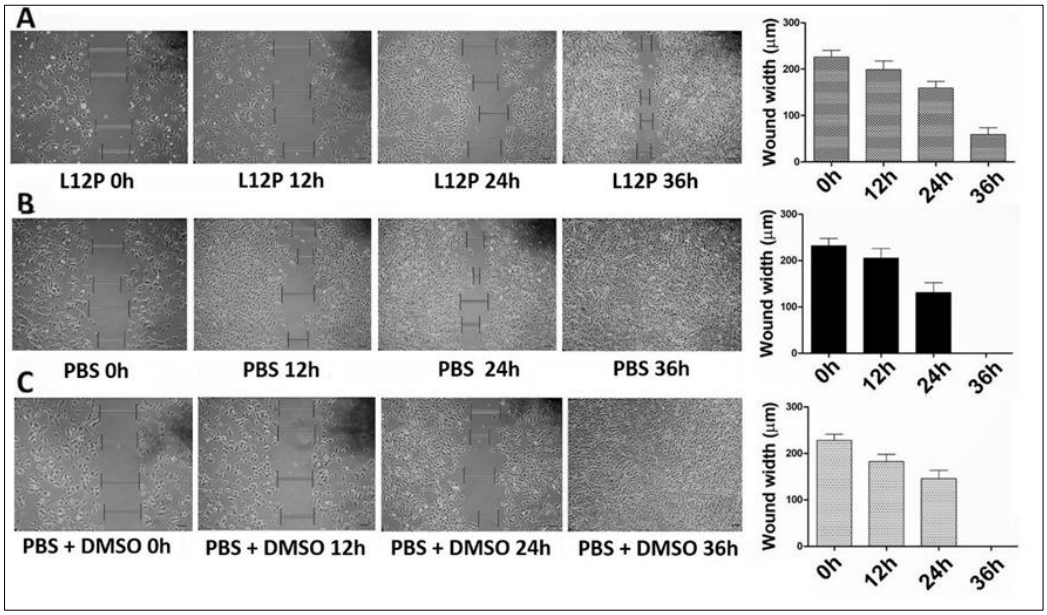

Figure 2 Wound-healing assay after L12P treatment shows impaired cell migration. The migration ability of tumor cells was determined using the wound-healing assay. The L12P was added at 1 mM and images were taken at regular intervals of 12 h; the graphics represents the area between cells. Bar is 150 μm. Graph of area, black PBS control, gray treated L12P and light gray PBS + DMSO control. The area of cell migration was measured in μm by ImageJ. (A) Using peptide L12P, 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; (C) Using PBS + DMSO, the wound was completely healed after 36 h

The wound-healing assay was used to answer the question, if the L12P 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. 2A); in contrast after 36 h, PBS control cells migrated to completely and fill the wound gap (Figure. 2 B), and control PBS+DMSO behaved like the PBS control, with normal cell migration (Figure. 2 C) thus, we can detect a direct effect of the peptide on cell migration.

As last experiment we used flow cytometry to analyze the cell cycle using propidium iodide DNA staining. We observed a difference between L12P and control PBS+DMSO treated cells, where was verified an increase of S phases and consequently decrease in the G2M phase (Figure. 3 A and B).

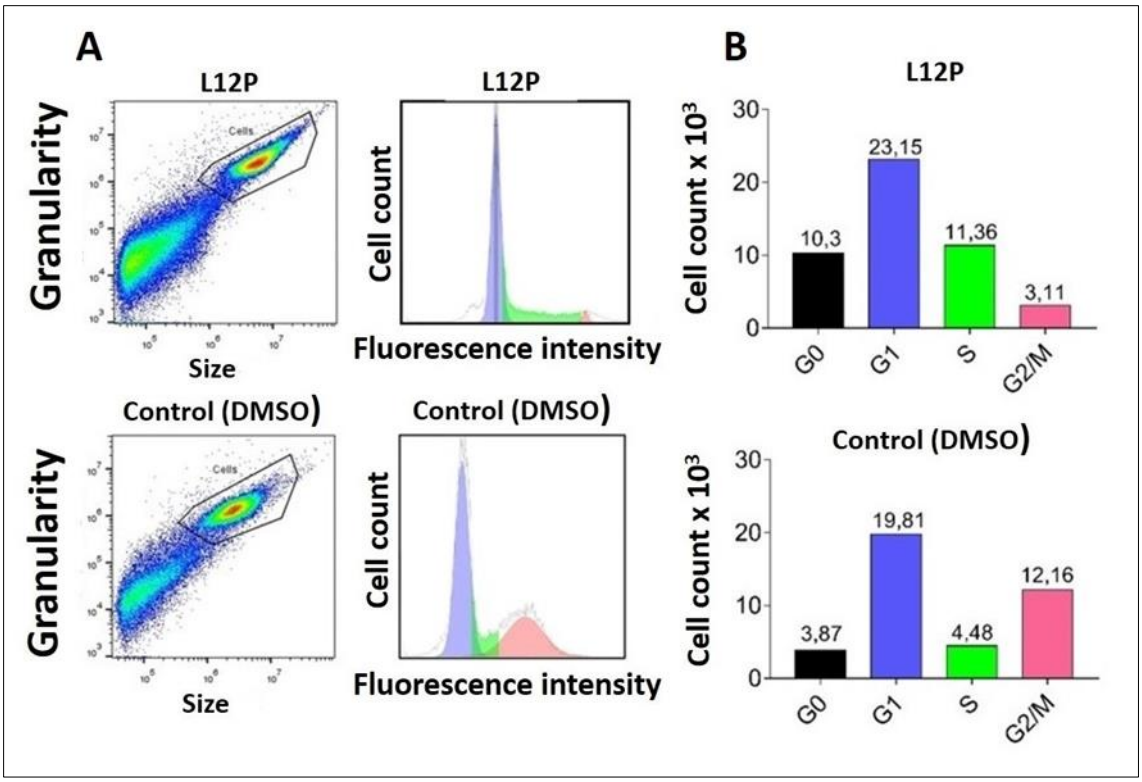

Figure 3 Cell cycle modulation with L12P treatment. 10^6 B16F10 cells were treated with L12P and analyzed by flow cytometer after incubation with propidium iodide (PI) solution. (A) PI staining shows different intensities in the population of L12P treated cells with difference in cell cycle pattern in comparison to PBS + DMSO control. (B) Number of cells in each phase of the cycle showed L12P treated and control PBS + DMSO in G1, S and G2 cell cycle phases. The graphs and analyzes were made using FlowJo software

4. Discussion

Firstly, to analyze the live and dead cells was used Trypan blue exclusion staining, the L12P have a toxic effect only at doses superior at 1.5 mM (data no shown); so, the max 1 mM dose used not cause unspecific death and is safe to answer the experimental questions. The MTT result indicates that the L12P acts on cell viability, thus in future we intend do an additional test of cell proliferation in B16F10-Nex2 cells.

The wound-healing assay shows that L12P has inhibition in tumor cell migration with a direct effect of peptide in cell migration, so we aim for a future clonogenic assay to observe the toxic effect of the peptide in B16F10-Nex2 cells.

Lastly, the effect on cell cycle could be explained by the interaction of L12P with cycle molecules, thereby the tumor cells in L12P 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) [5]; in the future this result can be elucidated by Western blotting of the molecules involved in the cell cycle.

5. Conclusion

To conclude we verified that L12P acts on cell viability, the peptide has inhibition in tumor cell migration and in cell cycle causing an increasing of S phase with decreasing in G2M phase and probably because of that causing a delay in the cell cycle thus elucidating observed data in MTT and cell migration. These results support the L12P display a therapeutic activity essential to obtain future antitumoral test in animal models.

Finally, how this study will benefit to the society and way forward obtaining peptide sequences that have therapeutic action against melanoma and many other types of cancer.

Compliance with ethical standards

Acknowledgments

This work was supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) through doctoral and career fellowships. We also thank Professor Luciano Polonelli from the University of Padua in Italy.

Disclosure of conflict of interest

The authors declare that there is no conflict of interest.

Authors Contributions

E A X, F C M and L R R G T conceived the study, E A X and F C M conducted the experiments E A X and F C M analyzed the data, E A X wrote the manuscript, all authors read and approved the final version.

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