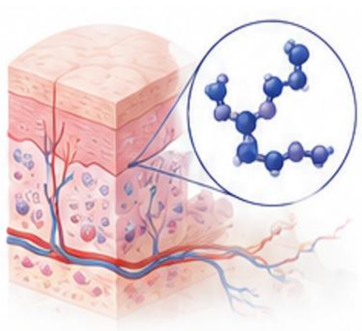
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Multifunctional Short Chimeric Peptides: Potential Antimicrobial Activity and Enhanced Cell Proliferation for Tissue Healing

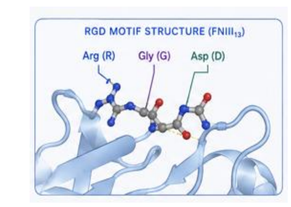
Naiara Maia Pereira, **Reinaldo Marchetto**

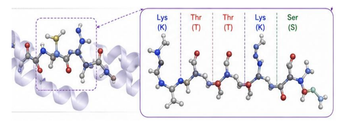
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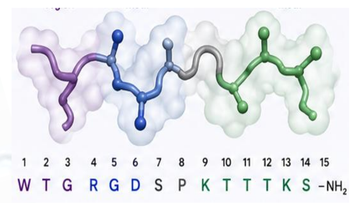
INTRODUCTION



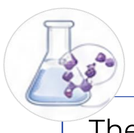
Tissue healing is a complex biological process that can be impaired or delayed by several factors, such as hypoxia, ischemia, altered cellular response, bacterial colonization and defects in collagen synthesis, extending the healing period from a few weeks to many years[1]. There are four phases of healing, each playing a crucial role in restoring tissue function: hemostasis, inflammation, proliferation, and maturation. Short peptides can be combined to control and enhance some of these phases, resulting in faster healing processes in a more controlled environment[1,2]. Studies have shown that short sequences of peptides can inhibit the microbial growth of some species and stimulate the Extracellular Matrix to increase the cell proliferation, supporting some events of the healing processes and making them more effective[2,3,4]. In this context, we designed and synthesized a series of novel chimeric peptides with structures that combines fibronectin fragments and a sub-fragment of type I collagen propeptide, to achieve enhanced cell proliferation, collagen synthesis and microbial control. The peptides were chemically synthesized by Solid Phase methodology, purified by RP-HPLC and characterized by mass spectrometry (ESI-MS). Cytotoxicity analysis was performed by Hemolytic Assay against human blood cells and by MTT assay against MRC-5 (ATCC CCL-171) and keratinocyte (BCRJ 0341) cell lines. Microbial growth inhibition was evaluated by the minimum inhibitory concentration (MIC) assay against Staphylococcus aureus (ATCC 14458) and Escherichia coli (ATCC 43895). The NMB peptide showed no significant cytotoxicity in fibroblasts or keratinocytes. In addition, low hemolytic activity was observed indicating a safety profile that is favorable in the context of wound healing. Finally, NMB peptide significantly reduced microbial growth for both strains analyzed, by approximately 50% at all tested concentrations. Our findings indicate that the NMB peptide combines antimicrobial activity with low cytotoxicity and hemolytic effect, making it a promising candidate for future research as a wound-healing compound for the treatment of wounds





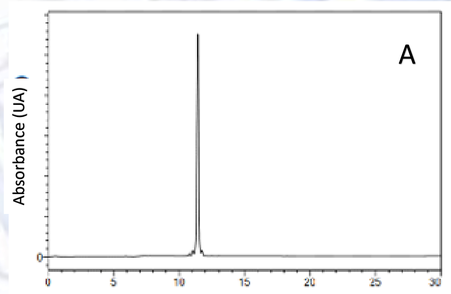


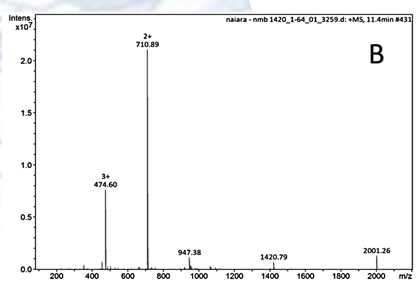
MATERIAL, METHODS AND RESULTS



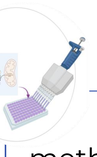
The peptide NMB, WTGRGDSPKTTKS, was synthesized by solid-phase methodology, using Fmoc/tBu Chemistry with a Fmoc-Ser(tBu)-Wang resin (0.33 mmol g^{-1}) and DIC/HOBt activation. After deprotection and cleavage with TFA/water/TIS (95:2.5:2.5) for 120min at RT, the peptide was precipitated, solubilized and dried under vacuum. Analysis and purification was performed by RP-HPLC and the identity confirmed by ESI-MS.

Calculated MW (g.mol-1)	ES+	ES m/z (g.mol-1)	Obtained MW (g.mol-1)
1.420,53	+2	710,89	1420,46
	+3	474,6	

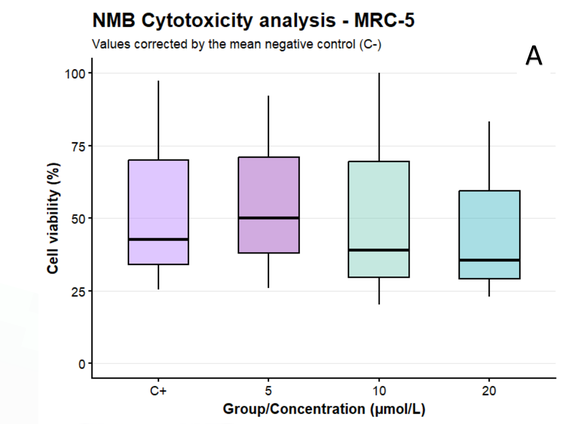


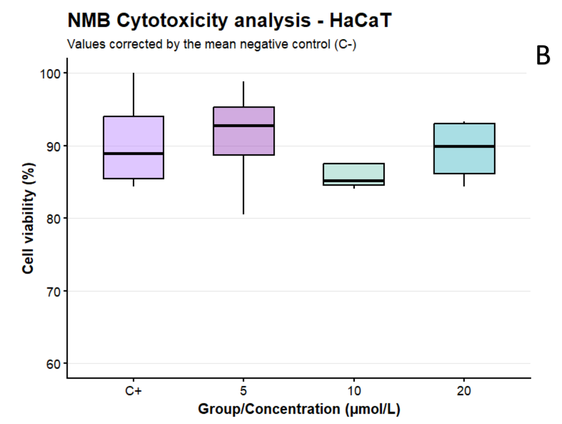


Analytical-scale chromatographic profiles of the NMB peptide obtained after purification (A). Separation was performed on a Jupiter C18 reverse-phase column (250 × 4.6 mm, 5 µm, 300 Å; Phenomenex). A linear gradient from 5 to 95% solvent B (A: water containing 0.045% TFA; B: acetonitrile containing 0.036% TFA) was applied over 30 min at a flow rate of 1.0 mL min^{-1}, with UV detection at 220 nm. (B) Mass spectra obtained via ESI-MS confirming the pureness of the peptide obtained



Cytotoxicity analysis was performed using the MTT colorimetric method for 24h, using fibroblasts MRC-5 (ATCC CCL-171) and HaCaT cells, immortalized human keratinocytes (BCRJ 0341). To perform the MTT assay, the supernatants were removed and discarded. So, 100 µl of sterile PBS buffer was added to the wells to remove dead cells and other metabolic residues present. Next, 180 µL of DMEM and PBS (1:4) solution were added, followed by the addition of 20 µL of MTT solution at 1.0 $mg.mL^{-1}$. The plates were kept in incubation for 4 hours at 37°C and 5% CO_2. Finally, the supernatants were discarded and 100 µL of a mixture 1:1 of isopropyl alcohol and dimethyl sulfoxide was added to each well to dissolve the formed crystals, keeping the set protected from light and under agitation for 1 hour. The absorbance was read using a microplate reader spectrophotometer at 540 nm.

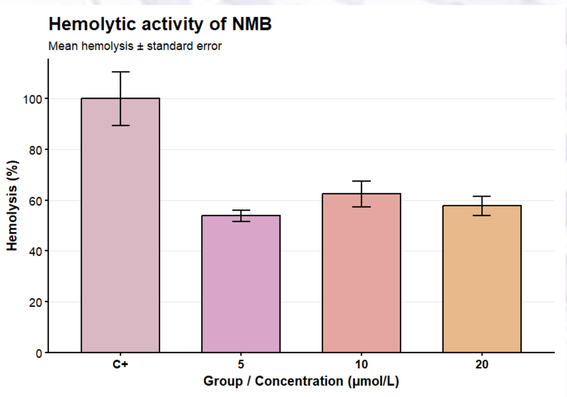




Cell viability of fibroblasts (A) and keratinocytes (B) treated with NMB at different concentrations. Cell viability was evaluated by the MTT assay after treatment with NMB at 5, 10, and 20 µmol/L, compared with the untreated control (C+). Values were corrected using the cytotoxicity control (C−) and normalized to the highest value obtained, which was set as 100% viability. Data are presented as boxplots from three independent experimental replicates, with technical duplicates averaged within each replicate. No statistically significant differences were observed among the groups for either cell line (one-way ANOVA followed by Tukey's multiple comparisons test, $p > 0.05$).



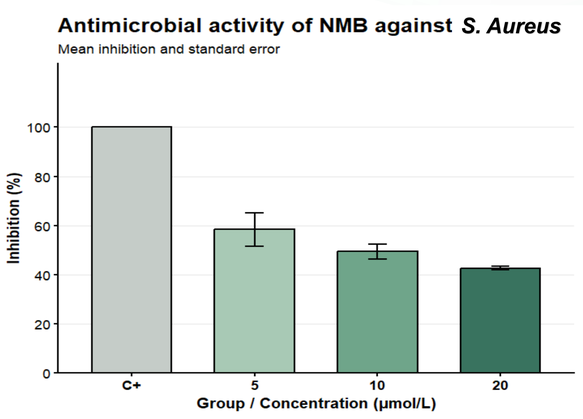
Hemolytic analysis were performed using washed erythrocytes isolated from human blood cells. To perform the assay, 100 µL of washed erythrocytes solution were added to a microtube with 100 µL of the peptide solution and incubated under 37°C for 1 hour. After that, the microtubes were centrifuged for 3 minutes at 3000 rpm and 100 µL of the supernatant were transferred to a 96 well plate and placed in a microplate reader to measure absorbance at 540 nm. The positive control was washed erythrocytes solution with an 1% triton solution and negative control was the equivalent number of cells with PBS, pH 7,4.



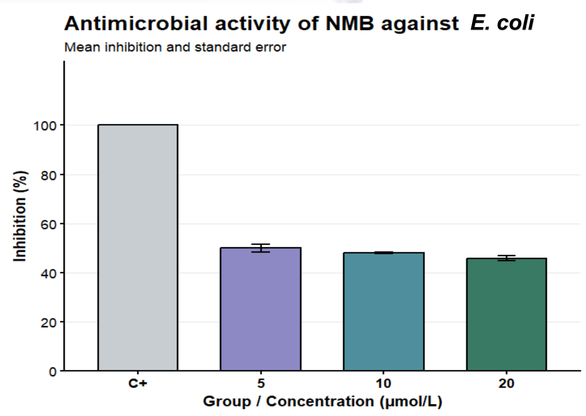
Hemolytic activity of NMB at 5, 10, and 20 µmol/L compared with the positive control (C+). Hemolysis was normalized to the positive control, which was defined as 100% hemolysis. Data represent three independent experiments. NMB exhibited significantly lower hemolysis than the positive control at all concentrations tested, respectively for 5 µmol/L, 10 µmol/L and 20 µmol/L, $p = 0{,}0037$; $p = 0{,}0123$ and $p = 0{,}0062$, although one-way ANOVA ($F_{3,8} = 11.48$, $p = 0.00286$) and Tukey's multiple comparisons test ($p < 0.05$), have shown no significant differences among NMB concentrations ($p > 0.05$).



Antimicrobial activity was performed using the plate microdilution method with modifications described by the Clinical and Laboratory Standards Institute (CLSI – M7-A10, 2015 and M27-A3, 2017). After initial growth, a cellular suspension was adjusted to McFarland scale 0.5 (10^8 CFU/mL) and diluted to 10^7 CFU/mL. For peptide analysis, 80 µL of culture media and 100 µL of peptide solution were added to each well, followed by 20 µL of microbial suspension, and incubated for 24 hours at 37°C. Positive controls were prepared similarly, using microorganisms and culture media, while negative controls included media-only. After the incubation period, bacterial growth was assessed by measuring the optical density at 595 nm using a microplate reader spectrophotometer.



NMB reduced *S. aureus* growth by approximately 42–57% relative to the positive control across all concentrations tested (5, 10, and 20 µmol/L). All NMB concentrations differed significantly from the positive control (one-way ANOVA, $F(3,8) = 48.73$, $p < 0.001$; Tukey's multiple comparisons test, all $p < 0.001$). No significant differences were observed among NMB concentrations (all $p > 0.05$).



NMB reduced *E. coli* growth by approximately 50–54% relative to the positive control at the concentrations tested (5, 10, and 20 µmol/L). All NMB concentrations differed significantly from the positive control (one-way ANOVA, $F(3,8) = 739.4$, $p < 0.001$; Tukey's multiple comparisons test, all $p < 0.001$), whereas no significant differences were observed among NMB concentrations (all $p > 0.05$).

CONCLUSION

The fibronectin- and collagen-derived peptides were successfully obtained, containing the RGD and KTTKS sequences, and showed no significant cytotoxicity in fibroblasts or keratinocytes at any of the tested concentrations (5–20 µmol/L). In addition, low hemolytic activity was observed compared to the control, indicating a safety profile that is favorable in the context of wound healing, as it helps control bleeding from an acute wound and promotes the initiation of the healing process, possibly triggering the homeostasis. Finally, the peptide significantly reduced microbial growth for both strains analyzed, by approximately 50% at all tested concentrations. Thus, the findings indicate that the NMB peptide combines promising antimicrobial activity with low cytotoxicity and hemolytic effect, making it a promising candidate for future research as a wound-healing compound for the treatment of wounds.

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