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Faculdade de Ciências Farmacêuticas

Faculdade de Farmácia

Cesar Augusto Roque Borda

In vitro and *in vivo* studies of antimicrobial peptide B1CTcu5 analogs encapsulated in colon-specific microparticles against *Mycobacterium tuberculosis*

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In vitro and *in vivo* studies of antimicrobial peptide B1CTcu5 analogs encapsulated in colon-specific microparticles against *Mycobacterium tuberculosis*

Cesar Augusto Roque Borda

M.Sc. in Animal Science

B.Sc. of Biotechnology Engineering

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Thesis specially elaborated under a double PhD degree agreement between the Sao Paulo State University and the University of Lisbon for the attainment of the degree of Doctor in Sciences, specializing in Biotechnology; and in Pharmacy, specializing in Microbiology, respectively.

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Estudos *in vitro* e *in vivo* de análogos do peptídeo antimicrobiano B1CTcu5 encapsulados em micropartículas colón-específicas frente ao *Mycobacterium tuberculosis*

Discente: **Cesar Augusto Roque Borda**

Orientadores: **Prof. Doutor. Fernando Rogério Pavan (UNESP)**

Prof. Doutor. João Perdigão (ULisboa)

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“Entre ser o no ser, Yo soy”

Teresa

“Só existem dois dias do ano em que você não pode fazer nada: um se chama ontem e outro amanhã”.

Dalai Lama

RESUMO

A tuberculose (TB) permanece como um dos principais desafios de saúde pública global, agravado pela emergência de cepas de *Mycobacterium tuberculosis* (MTB) resistentes aos fármacos disponíveis. Diante desse cenário, esta tese teve como objetivo geral o desenvolvimento, a caracterização e a aplicação de peptídeos antimicrobianos e sistemas avançados de liberação de fármacos como estratégias alternativas e complementares no combate à tuberculose e a infecções bacterianas associadas. O Capítulo 1 apresenta as considerações gerais da tese, incluindo a contextualização do problema, os objetivos do trabalho e uma revisão crítica da literatura, abordando os limites das terapias convencionais, o potencial dos peptídeos antimicrobianos e o papel de sistemas de liberação no aumento da eficácia e seletividade terapêutica. O Capítulo 2 descreve a etapa de descoberta e desenho racional de peptídeos antimicrobianos com atividade antituberculosa. Uma biblioteca de análogos peptídicos foi gerada por síntese iterativa em bateladas, resultando na identificação do peptídeo CR22DK-S18G, que apresentou concentração inibitória mínima (CIM) de 1,2 µg/mL contra *Mycobacterium tuberculosis*, com seletividade elevada e baixa citotoxicidade. O capítulo aborda de forma abrangente a síntese peptídica, a avaliação biológica, estudos de seletividade, mecanismos de ação e modulação da resposta imune. O Capítulo 3 é dedicado à investigação das interações dos peptídeos antimicrobianos com a membrana micobacteriana. Foram integradas abordagens *in silico*, incluindo docking molecular e simulações de dinâmica molecular, com ensaios experimentais, permitindo compreender a interação dos peptídeos com membranas ricas em ácidos micólicos e correlacionar esses dados com a atividade antimicrobiana observada. O Capítulo 4 explora o uso de sistemas de liberação direcionados ao cólon, baseados em micropartículas de alginato com dupla cobertura de quitosana e HPMCAS, carregadas com peptídeos antimicrobianos. Os resultados demonstram proteção do peptídeo ao longo do trato gastrointestinal e eficácia na prevenção de infecções intestinais, destacando o potencial dessas formulações para aplicações específicas no ambiente entérico. O Capítulo 5 aborda sistemas de liberação baseados em auto-organização, utilizando micropartículas de alginato-pectina contendo peptídeos antimicrobianos autoassociados e resveratrol. Este capítulo demonstra a obtenção de sistemas com atividade antimicrobiana e anti-

inflamatória combinada, evidenciando a relevância da auto-montagem peptídica para aplicações terapêuticas multifuncionais. O Capítulo 6 apresenta aplicações nanotecnológicas de peptídeos antimicrobianos contra *Mycobacterium tuberculosis*, com foco em nanopartículas de quitosana modificadas com N-acetilcisteína funcionalizadas com peptídeos. As formulações desenvolvidas foram capazes de potencializar a atividade de fármacos contra isolados clínicos de MTB, demonstrando sinergismo e relevância translacional. Por fim, o Capítulo 7 reúne as perspectivas e conclusões gerais da tese, integrando os principais achados e discutindo o potencial dos peptídeos antimicrobianos associados a sistemas avançados de liberação de fármacos como plataformas terapêuticas promissoras. A tese estabelece bases sólidas para o desenvolvimento futuro de conjugados peptídicos multifuncionais e estratégias racionais voltadas ao enfrentamento da tuberculose e de infecções bacterianas resistentes.

Palavras-chave: peptídeos antimicrobianos; descoberta de fármacos; liberação controlada; tuberculose.

ABSTRACT

Tuberculosis (TB) remains one of the major global public health challenges, exacerbated by the emergence of drug-resistant strains of *Mycobacterium tuberculosis* (MTB). In this context, the overall objective of this thesis was the development, characterization, and application of antimicrobial peptides and advanced drug delivery systems as alternative and complementary strategies for the treatment of tuberculosis and associated bacterial infections. Chapter 1 presents the general considerations of the thesis, including the problem background, the objectives of the study, and a critical review of the literature, addressing the limitations of conventional therapies, the potential of antimicrobial peptides, and the role of delivery systems in enhancing therapeutic efficacy and selectivity. Chapter 2 describes the discovery and rational design of antimicrobial peptides with antitubercular activity. A library of peptide analogues was generated through iterative batch synthesis, leading to the identification of the peptide CR22DK-S18G, which exhibited a minimum inhibitory concentration (MIC) of 1.2 µg/mL against *Mycobacterium tuberculosis*, with high selectivity and low cytotoxicity. This chapter comprehensively addresses peptide synthesis, biological evaluation, selectivity studies, mechanisms of action, and immune response modulation. Chapter 3 is dedicated to the investigation of antimicrobial peptide interactions with the mycobacterial membrane. *In silico* approaches, including molecular docking and molecular dynamics simulations, were integrated with experimental assays, enabling the elucidation of peptide interactions with mycolic acid-rich membranes and the correlation of these interactions with the observed antimicrobial activity. Chapter 4 explores colon-targeted delivery systems based on alginate microparticles with a double coating of chitosan and HPMCAS, loaded with antimicrobial peptides. The results demonstrate peptide protection throughout the gastrointestinal tract and efficacy in preventing intestinal infections, highlighting the potential of these formulations for site-specific applications in the enteric environment. Chapter 5 addresses self-assembly-based delivery systems, employing alginate–pectin microparticles embedding self-associated antimicrobial peptides and resveratrol. This chapter demonstrates the development of systems with combined antimicrobial and anti-inflammatory activity, underscoring the relevance of peptide self-assembly for multifunctional therapeutic applications. Chapter 6 presents

nanotechnological applications of antimicrobial peptides against *Mycobacterium tuberculosis*, focusing on N-acetylcysteine-modified chitosan nanoparticles functionalized with peptides. The developed formulations were able to potentiate drug activity against clinical MTB isolates, demonstrating synergistic effects and translational relevance. Finally, Chapter 7 brings together the general perspectives and conclusions of the thesis, integrating the main findings and discussing the potential of antimicrobial peptides combined with advanced drug delivery systems as promising therapeutic platforms. Overall, this thesis establishes solid foundations for the future development of multifunctional peptide conjugates and rational strategies aimed at combating tuberculosis and drug-resistant bacterial infections.

Keywords: antimicrobial peptides, control release, drug discovery, tuberculosis.

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CHAPTER 1

1. GENERAL CONSIDERATIONS

1.1. INTRODUCTION

Antimicrobial peptides (AMPs) are a class of natural molecules that have antimicrobial activity against a wide variety of microorganisms, including bacteria, fungi, and viruses. AMPs are produced by a wide variety of organisms, from bacteria and fungi to plants and animals, including humans (Haney, Mansour e Hancock, 2022). These molecules are one of the oldest and most important defenses in innate immunity against microbial infections. AMPs are short and cyclic or linear peptide structures characterized by having a positive charge and an amphipathic structure, which allows them to interact with the cell membranes of microorganisms and cause their death. AMPs act in various ways, such as disrupting the integrity of the cell membrane, altering nutrient transport, and promoting cell lysis (Hancock e Sahl, 2006; Pontes *et al.*, 2022a).

AMPs have been the subject of great interest in biomedical and pharmaceutical research due to their antimicrobial activity and their potential to develop new antimicrobials. In addition, it has been shown that AMPs also have other biological activities, such as immunomodulation, wound healing, and antitumor activity (Polinário *et al.*, 2023). Although there is a wide variety of AMPs with different structures and properties, most of them share certain common characteristics. Some examples of antimicrobial peptides include nisin, colistin, magainin, melittin, and defensin (Mahlapuu *et al.*, 2016; Silveira, Roque-Borda e Vicente, 2021b).

Brevinin peptides are a class of antimicrobial peptides that are found in the skin secretions of frogs. These peptides have been studied extensively for their antimicrobial properties and have been found to have activity against a wide range of microorganisms, including bacteria, fungi, and viruses (Conlon *et al.*, 2007). Brevinin peptides are typically small molecules, consisting of between 18 and 39 amino acids, and they adopt a characteristic alpha-helical structure. This structure is believed to be important for their ability to disrupt the cell membranes of microorganisms and thereby kill them. In addition to their antimicrobial activity, brevinin peptides have also been found to have other biological effects, including anti-inflammatory and analgesic activities (Ghavami *et al.*, 2008). Several studies have investigated the potential of brevinin peptides as therapeutic agents for various infections, including tuberculosis. It has been shown that some brevinin peptides have significant antimicrobial activity

against *Mycobacterium tuberculosis* (MTB), the bacterium that causes tuberculosis, and may be a promising alternative to conventional antibiotics in the treatment of this disease (Abraham *et al.*, 2020).

The antimicrobial activity of brevinin peptides against MTB, the bacterium that causes tuberculosis, has been investigated in several studies. It has been shown that some brevinin peptides have antimicrobial activity against MTB and may be a promising alternative to conventional antibiotics in the treatment of tuberculosis. A 2015 study evaluated the antimicrobial activity of various brevinin peptides against MTB and found that some of them had significant activity against the bacterium and these peptides did not show toxicity to human cells (Shah *et al.*, 2022). These findings suggest that brevinin peptides have the potential to be a therapeutic alternative against tuberculosis and deserve further research in this area (Kumar *et al.*, 2015). For these reasons aforementioned, the present study is based on the drug discovery and application of most promising B1CTcu5 antimicrobial peptide analog, chosen for its potential antimicrobial activity against MTB and its *in vivo* administrations.

1.2. OBJECTIVES

To study the efficacy and translational potential of novel AMP analogs in tuberculosis therapy by combining *in vitro* and *in vivo* evaluations with nanotechnology-based approaches.

1.2.1. Specific Objectives

- ✓ Synthesize, purify and characterize the performance of each batch of AMP
- ✓ To evaluate the antimicrobial and antimycobacterial activity of each synthesized AMP
- ✓ To evaluate the cytotoxicity and hemolysis of each AMP
- ✓ Studying the activity against efflux bombs in MTB
- ✓ To study the mechanisms of action and cell penetration of the selected AMPs by microscopy and circular dichroism
- ✓ To study the activity of AMPs in intramacrophagic infections
- ✓ To evaluate the anti-MTB activity in *Galleria mellonella* as alternative model
- ✓ To validate the mechanism of action using dynamic studies.
- ✓ To evaluate the effectivity of AMPs in nanosystems against MTB.

CHAPTER 7

PERSPECTIVES AND CONCLUSIONS

Conclusions

This thesis demonstrates that antimicrobial peptides constitute a viable and versatile platform for the development of alternative therapeutic strategies against *Mycobacterium tuberculosis*, particularly in the context of drug-resistant infections. Through an integrated approach combining peptide discovery, mechanistic investigation, and formulation into advanced delivery systems, this work provides a comprehensive assessment of peptide-based interventions across multiple biological and technological levels. Among the peptide candidates investigated, the optimized analogue CRDK22-s18G exhibited potent antimycobacterial activity, improved efficacy compared to its parent compound B1CTcu5, and a favorable safety profile, including low cytotoxicity and efficient macrophage penetration. In addition to its direct antimicrobial activity, evidence of efflux pump modulation supports a multifactorial mode of action, which may contribute to enhanced activity against resistant mycobacterial strains. These findings highlight the relevance of rational peptide optimization for improving both efficacy and selectivity.

Beyond peptide discovery, this thesis emphasizes the critical role of delivery systems in maximizing therapeutic performance. Encapsulation within liposomal, polymeric, and nanostructured platforms improved peptide stability, bioavailability, and intracellular targeting, addressing key limitations commonly associated with peptide-based therapeutics. The evaluation of colon-targeted systems, self-assembling peptide formulations, and nanoparticle-based strategies further demonstrates the adaptability of antimicrobial peptides to diverse delivery approaches and clinical contexts. Collectively, the results presented herein underscore the potential of antimicrobial peptides, when combined with rational design and appropriate delivery technologies, to complement existing tuberculosis therapies. Rather than proposing a single therapeutic solution, this work establishes a modular and translational framework that can be extended to other peptide scaffolds, drug combinations, and infectious disease settings. As such, this thesis contributes to the advancement of peptide-based strategies aimed at overcoming the current limitations of conventional antitubercular treatments and addressing the ongoing challenge of drug-resistant tuberculosis.

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