



## Short Communication

Potent cationic antimicrobial peptides against *Mycobacterium tuberculosis* in vitroSara Silva<sup>a,b,c</sup>, Anabela Santos-Silva<sup>d</sup>, José Manuel Correia da Costa<sup>d,e</sup>, Nuno Vale<sup>a,b,c,f,\*</sup><sup>a</sup> Laboratory of Pharmacology, Department of Drug Sciences, Faculty of Pharmacy, University of Porto, Porto, Portugal<sup>b</sup> Institute of Molecular Pathology and Immunology of the University of Porto (IPATIMUP), Porto, Portugal<sup>c</sup> Instituto de Investigação e Inovação em Saúde (i3S), University of Porto, Porto, Portugal<sup>d</sup> National Health Institute Dr. Ricardo Jorge (INSA), Porto, Portugal<sup>e</sup> Center for the Study of Animal Science, CECA-ICETA, University of Porto, Porto, Portugal<sup>f</sup> Department of Molecular Pathology and Immunology, Abel Salazar Biomedical Sciences Institute (ICBAS), University of Porto, Porto, Portugal

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## ABSTRACT

**Background:** Tuberculosis (TB) is known to be one of the 10 causes of global death by infectious agents. The increasing numbers of multiple antibiotic resistance (MDR-TB) and cases of extensive resistance to antibiotics (XDR-TB) have led to the development of new and effective TB therapy. Cationic antimicrobial peptides (CAMPs) have emerged in the research as a safe and effective treatment against a variable range of bacterial and fungi pathogens, including *Mycobacterium tuberculosis* (*M. tuberculosis*).

**Method:** This study developed a new CAMP coupled with cinnamic acid derivatives, and studied the antimicrobial activity against clinical isolates of *M. tuberculosis* (H37Rv) and MDR-TB.

**Results:** All modified CAMPs showed enhanced activity against both *M. tuberculosis* strains and were capable of disrupting heavy clumping of mycobacteria in culture. In addition, all modified CAMPs were able to substantially inhibit the intracellular growth of both strains at low concentrations.

**Conclusions:** The characteristic properties of cinnamic acid + CAMP(n) successfully inhibited the growth of both clinical isolates *M. tuberculosis* and MDR-TB in vitro and have, for now, promising use as a drug adjuvant due to their effect on mycobacteria growth.

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## 1. Introduction

Tuberculosis (TB) is considered to be one of the top 10 leading global causes of disease and mortality due to an infection agent. In 2015, according to the World Health Organization report, 10.4 million new cases were registered and 1.8 million deaths were caused by TB (1.4 million TB and 0.4 million resulting from coexistence with HIV) worldwide [1]. Treatment of TB has become more difficult to achieve, due in part to the duration of therapy and the rising number of multiple drug resistant strains (multi-drug resistant TB (MDR-TB) and extensively drug resistant TB (XDR-TB) [2].

Cationic antimicrobial peptides (CAMP)-based therapies are interesting candidates as alternatives or adjuvants to antibiotic treatments [3–5], with a wide spectrum of activity [6,7]. The potential properties of cinnamic acids were recently suggested, and more articles are being published to show potential use in

cancer, malaria, diabetes, and TB [8,9]. In particular for TB, there is a possibility that it affects the biosynthesis of mycolic acids present in *Mycobacterium tuberculosis* (*M. tuberculosis*) [10,11]. The current study was interested in developing, for the first time, a new CAMP with cinnamic acids and evaluating the effect against TB and the potential cytotoxicity.

## 2. Methods

Cationic antimicrobial peptides were synthesised using standard manual Fmoc-SPPS with nine amino acid length, as previously reported [12]. In order to improve CAMP(n) activity, a modification in the N-terminal side was performed by joining a cinnamic acid derivate in the best five peptides from work by Ramón-García (Fig. 1; see also Supplementary material Sections 1 and 2). In vitro antimicrobial activity of these CAMPs against *M. tuberculosis* H37Rv strain and clinical isolate MDR-TB (resistant to isoniazid, rifampicin and streptomycin) were assayed by the Resazurin Microtiter Assay Plate (REMA) method and visualised on light microscope. Briefly: the CAMPs were tested in Middlebrook 7h9 medium supplemented with 10% OADC (oleic acid 0.6g, bovine

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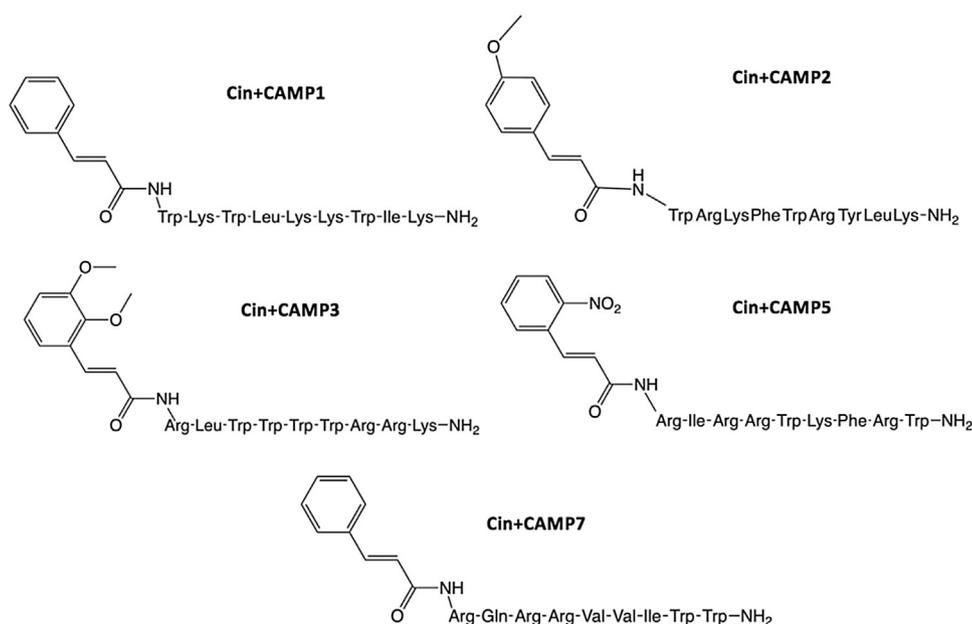


Fig. 1. Molecular structure of Cin + CAMP(n).

albumin 50 g, dextrose 20 g and a catalase 0.02 g) as serial two-fold dilution of the peptides (range 0.25–128  $\mu\text{g}/\text{mL}$ ) and 20  $\mu\text{L}$  of bacterial suspensions were added to a final volume of 200  $\mu\text{L}$ ; the plates were incubated for 7 and 14 days, respectively, at 37  $^{\circ}\text{C}$  and then incubated with Resazurin for 1 day (a change from blue to pink indicated bacterial growth). The MIC95 and MIC50 were defined as the lowest concentration of peptides that prevented the growth of > 95% or 50% of the bacterial population compared with the growth control. The cytotoxicity of the all CAMPs was determined by MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) tetrazolium assay, which measured cell viability in macrophage-like THP-1 cells.

### 3. Results and discussion

All peptides that were tested in this study showed concentration-dependent antibacterial activity against two clinical strains of *M. tuberculosis* (H37Rv and MDR) (Supplementary Table S3). In general, all modified CAMPs demonstrated enhanced potency

when compared with CAMP(n) without modifications. Peptides with most pronounced activity against susceptible *M. tuberculosis* were Cin + CAMP1 and Cin + CAMP3 with a MIC95 of 44.33  $\mu\text{M}$  and 38.51  $\mu\text{M}$ , respectively (Supplementary Table S3). At low concentrations of peptide, inhibition of growth was observed with a decrease of 50% (MIC50) when compared with the control wells. A MIC50 of the most active peptide was 0.69  $\mu\text{M}$  Cin + CAMP1 followed by 2.41  $\mu\text{M}$  of Cin + CAMP3. When compared with the respective CAMP1 and CAMP3, the MIC50 was 6.09  $\mu\text{M}$  and 5.44  $\mu\text{M}$ , corresponding with a reduction of 88.7% and 55.7% of the concentration of MIC50 (Fig. 2A; Supplementary Table S4). Notably, against resistant strains of *M. tuberculosis* (MDR-TB) the antimicrobial potential of the Cin + CAMP1 was preserved with a MIC of 44.33  $\mu\text{M}$ . With the exception of Cin + CAMP5, one of the possible explanations for lower activity of Cin + CAMP5 compared with CAMP5 could be the association with aggregation properties after peptide coupling. As can be observed in Supplementary Table S4, the *o*-nitrocinnamic acid is a nitroaromatic compound, and the nitrogen atom in a nitro group is positively charged and the

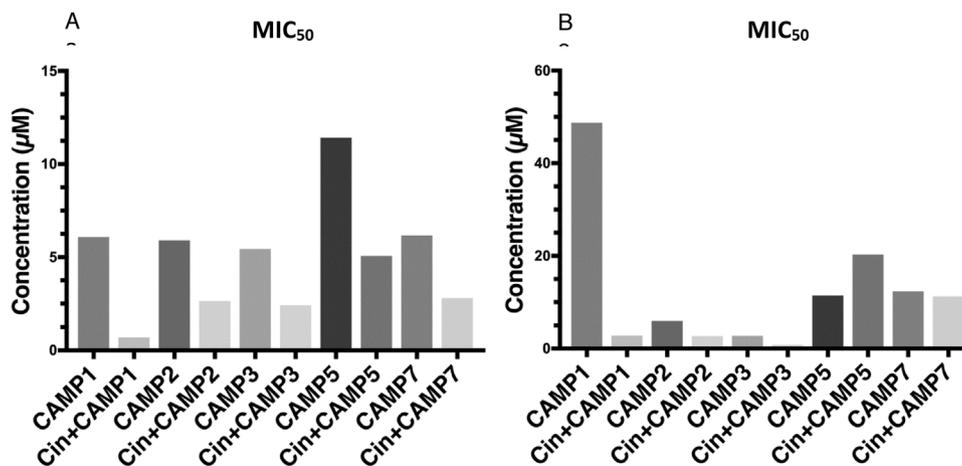
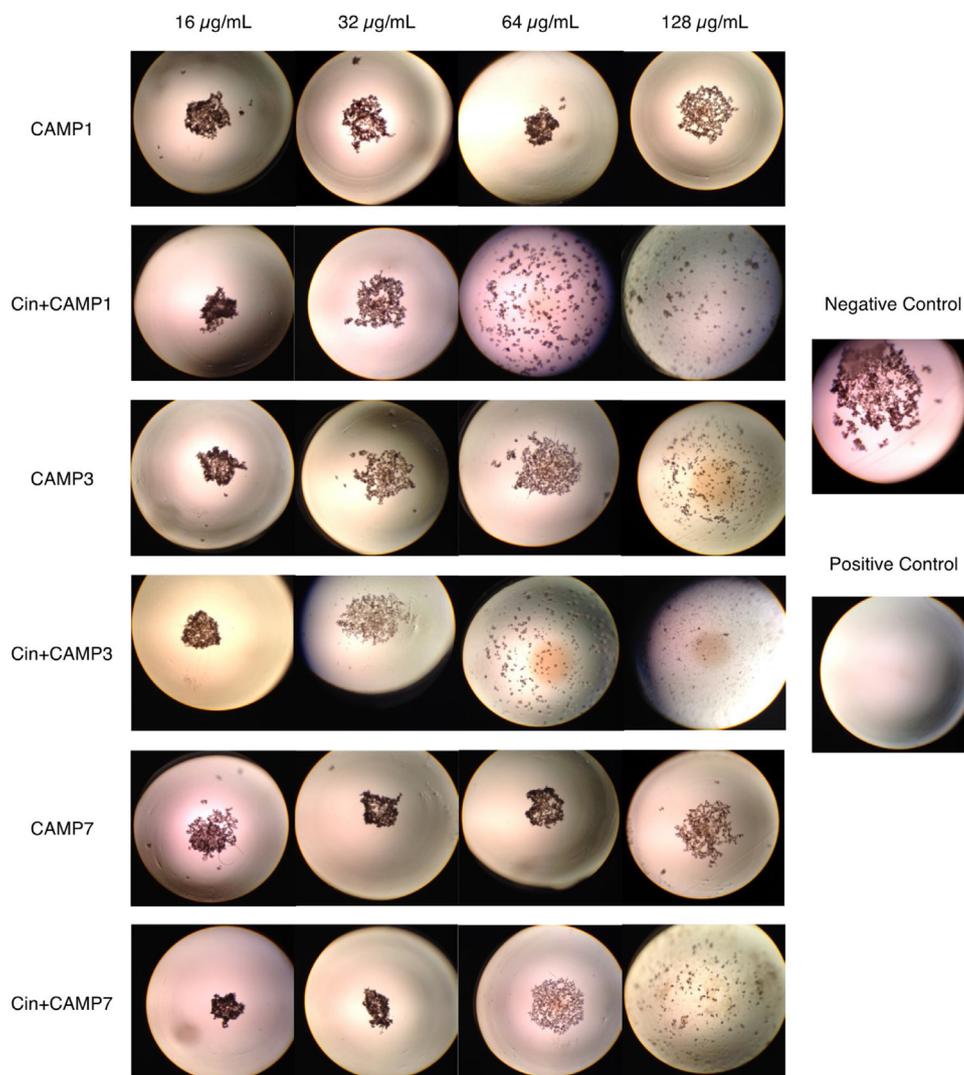


Fig. 2. MIC50 activities of CAMP(n) and Cin + CAMP(n) against (A) *Mycobacterium tuberculosis* H37Rv and (B) multi-drug resistant (MDR) *Mycobacterium tuberculosis* expressed in  $\mu\text{M}$ .



**Fig. 3.** In vitro antimicrobial activity of CAMP(n) against *Mycobacterium tuberculosis* clinical isolate susceptible strain H37Rv; representative light microscope images show the growth conditions of the bacteria at various concentrations of CAMP (1, 3 and 7) and Cin + CAMP (1, 3 and 7) after 7 days of incubation. Positive control with isoniazid.

two oxygen atoms share a negative charge. A nitroaromatic compound is a molecule in which one or more nitro groups are directly attached to an aromatic ring system. Chemically, the nitro group possesses a unique combination of properties: it is strongly electron-withdrawing, small, polar, and can form hydrogen bonds [13]. In addition, the nitro group can be bioactivated by enzymatic reduction to give reactive species. The current authors believe that these reactive species can also be responsible for the decrease in biological effects of *o*-nitrocinnamic acid with CAMP.

Regarding the improvement of MIC50 values observed for the *M. tuberculosis* MDR-TB, again, Cin + CAMP1 and Cin + CAMP3 showed the most effective inhibition of growth at low concentrations, with a MIC50 of 2.77  $\mu$ M and 0.60  $\mu$ M corresponding to a reduction of 94.3% and 77.9%, respectively, when compared with parental peptides (Fig. 2B; Supplementary Table S4).

The observed growth of cultures of susceptible *M. tuberculosis* taken through light microscope were also interesting. On cellular culture, the morphology of *M. tuberculosis* appeared as aggregated clumps of colonies. With the increased concentration of peptide, the growth of mycobacteria was further inhibited and an improvement in antimicrobial activities was observed (Fig. 3; see also Fig. S6 in the Supplementary material), which appeared to affect the composition

of the cell envelope and reduce virulence of *M. tuberculosis* in high concentrations of CAMP. This observation could be associated with the amphipathic properties of these newly modified peptides to have the ability to disturb the cell wall and possibly inhibit the biosynthesis of mycolic acids, which lead to dispersion and scattered-like effect in all treated cultures in high concentrations of CAMP. Another two studies have described the same physiologic behaviour by antimicrobial peptides [14,15]. Some cytotoxic effects were observed, with a range of 7.74–64.33  $\mu$ M (Table S2; Fig. S7 in Supplementary material), but low levels of cytotoxicity for the concentration of peptide used in the present study.

#### 4. Conclusion

In conclusion, this study demonstrated that Cin + CAMP(n) can successfully inhibit the growth of both clinical isolates *M. tuberculosis* and MDR in vitro. The characteristic proprieties of CAMPs may also be effectively used to separate aggregated *M. tuberculosis* by interacting with the mycobacteria surface. Even though some of the tested peptides were not effective in completely eradicating *M. tuberculosis*, some observational features in the cell culture and MIC50 values make Cin + CAMP(n) a

promising compound. Cin+CAMP1 and Cin+CAMP3 can be used as drug adjuvants, due to their effect on mycobacteria growth. Overall, this study demonstrated the prospective value of Cin+CAMP(n) as a new therapeutic approach against *M. tuberculosis* and MDR-TB. For future work, the next steps will require further optimisation of Cin+CAMP(n) with the possible association with nanoparticles or polyethylene glycol (PEG) delivery systems for drug delivery strategy. Moreover, possible evaluation of the synergy activity between Cin+CAMP(n) and conventional antibiotics could also be evaluated.

### Competing interests

None declared.

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### Ethical approval

Not required.

### Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.jgar.2019.04.018>.

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