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Interaction of blood components with cathelicidins and their modified versions

Kai Yu [a], Benjamin F.L. Lai [a], Jurnorain Gani [d], Ralf Mikut [c], Kai Hilpert [d, **, 1], Jayachandran N. Kizhakkedathu [a, b, *, 1]

[a] Centre for Blood Research and Department of Pathology & Laboratory Medicine, University of British Columbia, Vancouver, British Columbia V6T 1Z3, Canada
[b] Department of Chemistry, University of British Columbia, Vancouver, British Columbia V6T 1Z3, Canada
[c] Karlsruhe Institute of Technology, Institute for Applied Computer Science, Hermann-von-Helmholtz-Platz 1, 76344 Eggenstein-Leopoldshafen, Germany
[d] Institute of Infection and Immunity, St George's University of London, Cranmer Terrace, London SW17 0RE, UK

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ABSTRACT

Cationic antimicrobial peptides (cAMPs) serve as effective components of the innate host defense against microbial infections. cAMPs often show broad-spectrum antimicrobial activity, but narrow-band activity is also observed. Despite their great potential, the polycationic nature of cAMPs could cause serious side effects once in the bloodstream which may limit their applications. However, there is very limited knowledge available on AMPs interaction with blood components in spite of the fact that the most likely route of administration to treat systemic microbial infections for these peptides is intravenous, where they immediately come in contact with all blood components. In order to evaluate the therapeutic potential of cAMPs as new alternative to antibiotics, we investigated the impact of cathelicidin related cAMPs on red blood cell lysis, aggregation, platelet activation, blood coagulation, and complement activation. The influence of cAMPs on blood depends on hydrophobicity and number of charges in the peptides. The hemolytic activity of cathelicidin (bactenecin) variants was much less than that of indolicidin due to their lower hydrophobicity. Except indolicidin, none of the peptides induce platelet activation. Some of bactenecin variants (R3, Sub3 and W3) with higher charge inhibited the blood coagulation. The cAMPs did not activate or inhibit complement at the concentrations studied, expect for the peptide (Sub3). Our data shows that it is important to investigate cAMP-based drug candidates regarding their interaction with blood components early on in the development process. We anticipate that this new knowledge on blood interaction of antimicrobial peptides will help to design peptides with a better therapeutic window and with less side effects.

1. Introduction

The 2014 WHO report on bacterial resistance showed that we indeed are in immediate danger to loose treatment options for several bacterial infections [1]. This problem gets intensified by the lack of economic incentive for large pharmaceutical companies to develop novel antibiotics. Antibiotics with novel modes of action, able to kill multi-drug resistant bacteria, are urgently needed. It has been shown that cationic antimicrobial peptides (cAMPs) are able to kill such multi-drug resistant bacteria efficiently and are therefore an interesting class to develop further into novel antimicrobials [2,3]. Challenging for the development into drugs is the fact that some natural occurring as well as artificial cAMPs can show toxic side effects, for example indolicidin and LL37 [4–6]. To treat systemic infections, peptides will be most likely applied intravenously and therefore encounter and interact with blood immediately. Understanding of their potential toxicity or unwanted side effects with human blood is therefore a critical step to design peptides with an optimal therapeutic window.

* Corresponding author. Centre for Blood Research and Department of Pathology & Laboratory Medicine, University of British Columbia, Vancouver, British Columbia V6T 1Z3, Canada.
** Corresponding author. Institute of Infection and Immunity, St George's University of London, Cranmer Terrace, London SW17 0RE, UK.
E-mail addresses: khilpert@sgul.ac.uk (K. Hilpert), jay@pathology.ubc.ca (J.N. Kizhakkedathu).
[1] Both authors contributed equally.

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cAMPs possess broad-spectrum bactericidal characteristics and display excellent antibacterial, antifungal, and even antiviral traits [2,3]. cAMPs are amphipathic peptides characterized by an overall positive charge of the molecule and a variable proportion of hydrophobic amino acids. The antimicrobial activity of cAMPs is due to their ability to disturb the bacterial membrane integrity via the electrostatic interaction of cationic AMPs with negatively charged bacterial membrane [7–9]. Despite their great potential, due to the polycationic nature, hemolytic activity and cytotoxicity of cAMPs could cause serious adverse effects [4–6]. cAMPs are attracted to bacterial surfaces by electrostatic interaction, but could directly interact with host cells and lyse them. Sometimes the toxicity associated with cAMPs is related to the inherent hydrophobicity combined with high dose to compensate for their relatively short half-life due to the rapid protease digestion [10,11], or peptide aggregation [12]. Several structure–activity relation studies with AMPs suggest a direct correlation between peptide hydrophobicity and hemolytic activity [13–15]. It has been shown that in some cases, increase in chain length leads to decreased antimicrobial activity and increased hemolytic activity. Optimum charge to hydrophobic ratio achieved by systematic substitution of arginine and tryptophan is necessary to maximize antimicrobial potency, while maintaining low cytotoxicity [16]. cAMPs have also shown to have impact on the blood coagulation and participate in the multiple aspects of immunity and trigger the immune system [17]. Thus it would be beneficial to optimize the peptide sequence (by varying the amino acid content, sequence and overall peptide length) to decrease the hemolytic activity, minimize its impact on blood coagulation and immune system while maintaining the antibacterial activity.

Besides cationic AMPs, there are number of reports in the literature which demonstrate the toxicity of polycationic compounds used for antimicrobial purposes. For example, quaternized poly(vinylpyridine) (PVP) [18], polyamidoamine (PAMAM) dendrimers [19,20], polynorbornene derivatives [21] and poly(-propyleneimine) [22]. However, they all caused red blood cell (RBC) hemolysis, platelet activation and platelet aggregation. Post modifications to tune the overall hydrophobicity or substituting of amino-groups with neutral or anionic moieties are often employed to increase the hemocompatibility [23,24]. More information on the correlation of chemistry of the cationic compounds with their interaction with blood is needed before they can be used safely and effectively in biomedical applications [25], where blood is involved.

In this manuscript we report a systematic study on interaction of natural and synthetic cAMPs, which are from the cathelicidin family [26] having different charges and hydrophobicity, with various blood components. As a pioneering study to get a first impression what cAMPs might have for an effect on different blood components we have chosen two natural cathelicidins and five variants of another cathelicidin (bactenecin). The selected cathelicidins are well studied and variants of indolicidin already were used in clinical trials [27]. They all comprise only of natural amino acids and are not stabilized by disulfide bonds. LL37 is a human cathelicidin and was intensively investigated regarding its antimicrobial as well as immunoregulatory effects and in consequence for the importance on different diseases [28]. LL37 is an α-helical peptide, 37 amino acids long, containing no tryptophan. Indolicidin is a bovine cathelicidin that shows antimicrobial activity against Gram-negative and -positive bacteria, fungi, protozoa and HIV-1 [4]. It is 13 amino acids long and comprises of five tryptophans, displaying therefore the highest proportion of tryptophan in a natural cAMP. Bactenecin is a 12mer cyclic bovine peptide that is also member of the cathelicidins. In order to avoid another layer of complexity in this study (dimer formation, different ratios of oxidized and reduced cysteines under different assay conditions) we used versions, were the cysteines are exchanged by alanines. This peptide is called Bac2A and demonstrated moderate activity against both Gram-negative and -positive bacteria [29–31] with low toxicity. In previous studies [30,31], Bac2A was modified in order to improve the antimicrobial activity. This resulted in different peptides; four were picked for this study, three with single substitutions (W3, R3, P7), one with multiple substitutions (Sub3). Peptide P7 was used as a negative control, since it lost all the antimicrobial activities but still has very similar biophysical parameters as Bac2A. The antimicrobial activity of the AMPs was tested against Gram-negative or Gram-positive bacterial strains, including *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhimurium*, *Staphylococcus epidermidis*, *Enterococcus faecalis* and the yeast *Candida albicans*. The blood interaction was investigated by their impact on hemolysis, RBC aggregation, platelet activation, blood coagulation and complement activation.

2. Materials and methods

2.1. Design and synthesis of peptides

All peptides were purchased by UMpep (Nordwestuckermark, Germany) and Kinexus (Vancouver, Canada). Peptides were synthesized on resin using standard Fmoc-strategy, cleaved, purified (>90% purity) with HPLC, confirmed with mass spectrometry, lyophilized and stored at −20 °C until use.

2.2. Analysis of antimicrobial activity

For the MIC determination, a broth dilution method [32], which is one of the most commonly used techniques to determine the MIC of antimicrobial agents was used. The protocol we used here has been adjusted for antimicrobial peptides and is based on the guidelines of the two established organizations and committees, the CLSI (Clinical and Laboratory Standards Institute) and EUCAST (European Committee for Antimicrobial Susceptibility Testing). Briefly, the MIC of the peptides was measured in Mueller Hinton (MH) medium, whereby the assay was performed in sterile 96-well round bottom polypropylene microtiter plates with an inoculum of $2–7 \times 10^5$ colony forming units (CFU) per ml. The plates was incubated at 37 °C for 12–15 h and the MIC was reported as the minimal peptide concentration at which no growth is observed. Bacterial strains *E. coli* UB1005 (F-, *nal*A37, *met*B1), wild-type *Salmonella enterica* spp. Typhimurium, wild-type *P. aeruginosa* H103, *E. faecalis* ATCC29212, *S. aureus* ATCC25923, a clinical isolate of *S. epidermidis*; and *C. albicans* were obtained from R.E.W. Hancock (Department of Microbiology and Immunology, University of British Columbia), whereas *S. epidermidis* originated from B. Dill (Department of Microbiology and Immunology, University of British Columbia) and *C. albicans* originates from D. Speert (Department of Pediatrics, University of British Columbia).

In addition, molecular descriptors that describe proportions of amino acids with cationic charges and hydrophobicity [33] were computed with the Peptide Extension of the Matlab toolbox GaitCAD [34]. These physiochemical properties rely on grouping all amino acids based on similar charges and hydrophobicities into 3 “terms” (low, medium, or high). In a next step, the proportion of cationic amino acids (high isoelectric point, term 3) and hydrophobic amino acids (term 1, low Hopp–Woods values) is counted for each sequence.

2.3. Blood component interaction studies

2.3.1. Blood collection

Blood from healthy consented donors was either collected into 3.8% sodium citrated tubes with a blood/anticoagulant ratio of 9:1 or sodium heparin (86 USP units) tubes at the Centre for Blood