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Short communication

Bactericidal oncocin derivatives with superior serum stabilities

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ABSTRACT

The proline-rich antimicrobial peptide oncocin is remarkably active in vitro against a number of important Gram-negative bacteria of concern to humans owing to their increasing resistance to antibiotics, i.e. Enterobacteriaceae (*Escherichia coli*, *Klebsiella pneumoniae* and *Enterobacter cloacae*) and non-fermenting species (*Acinetobacter baumannii* and *Pseudomonas aeruginosa*). Degradation of oncocin in mouse serum was investigated in this study. Several approaches to stabilise the main cleavage sites (C-terminal to Arg-15 and N-terminal to Arg-19) by substituting either or both arginine (Arg) residues with non-proteinogenic amino acids, i.e. α-amino-3-guanidino-propionic acid, homoarginine, nitro-arginine, *N*-methyl-arginine, β-homoarginine, D-arginine (D-Arg) or ornithine (Orn), were tested. These modifications were found to increase the half-life of oncocin in full mouse serum. For oncocin with two Orn residues in positions 15 and 19, the half-life in full serum increased from 25 min to 3 h. An increase of >8 h was observed for oncocin with two D-Arg residues at these same positions. The antibacterial activities of these modified sequences were slightly better than the original oncocin sequence. Moreover, the three most stable analogues were found to be bactericidal against *E. coli* and were not toxic to HeLa cells or haemolytic to human erythrocytes.

1. Introduction

Infectious diseases are a major cause of death worldwide. Despite the considerable progress made in hygiene and antibiotic treatment strategies during the last century, bacterial infections still contribute substantially to high rates of mortality and morbidity. What is perhaps even more worrying is that the incidence of serious bacterial infections caused by pathogens resistant to the current antibiotics is rapidly increasing. A recent report from the European Centre for Disease Prevention and Control indicated that an estimated three million hospitalised patients acquire nosocomial infections every year, accounting for ca. 50 000 deaths [1]. Similar numbers were estimated by the US Centers for Disease Control and Prevention (CDC) for the USA, accounting for more than US$3.5 billion in additional healthcare costs. According to the CDC, ca. 70% of these nosocomial infections are caused by bacteria resistant to at least one of the current antibiotics. Gram-negative bacteria account for ca. 40% of all nosocomial infections, nearly twice as many as those caused by Gram-positive bacteria [2]. Currently, three species of Enterobacteriaceae (*Escherichia coli*, *Klebsiella pneumoniae* and *Enterobacter cloacae*) and two non-fermenting species (*Acinetobacter baumannii* and *Pseudomonas aeruginosa*) are causing great concern due to the rapid spread of multiresistant or extremely resistant strains. A very recent and even more frightening development has been the incidence of pan-resistant *A. baumannii*, *E. coli* and *K. pneumoniae* strains reported in outbreaks in India, Pakistan, the UK and the USA that were uniformly susceptible only to polymyxin treatment [3,4]. Although prudent use of current antibiotics may slow down further resistance development, innovative antimicrobial drug classes with novel modes of action are urgently required. Inducible gene-encoded antimicrobial peptides (AMPs) may represent such a promising class of antibiotics. Whereas most AMPs act via disruptive lytic or pore-forming mechanisms directly on the bacterial membrane, only a few inhibit specific intracellular targets. The latter group appears especially promising as agents with which to treat systemic infections, as they will most likely not cross-react with the corresponding human proteins owing to low sequence homologies and structural differences [5].

Mammalian AMPs kill bacteria in vitro but also probably stimulate the innate immune system and might even link it to the adaptive immune system [6], which could cause severe problems if AMPs are administered at higher therapeutic doses. Thus, an ideal antimicrobial drug lead should be neither toxic to human cells nor modulate the human immune system. Insects are so evolutionarily removed from humans that their AMPs will most likely not modulate the human immune system. Thus, insect-derived, proline-rich

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AMPs, which are neither toxic to human cell lines nor haemolytic, appear to meet all the requirements of an ideal antibiotic.

In light of these facts, several laboratories have recently designed and optimised novel proline-rich AMP analogues with interesting antibacterial activities and with no toxic effects on mammalian cell lines or erythrocytes [7–9]. The high proline content also provides fairly good stability against proteolytic degradation in mammalian serum, which can be even further reduced by stabilising the cleavage sites. Recently, the efficiency of these optimised peptides was also proven for mouse models with systemic infections of *E. coli*, *A. baumannii* and *Salmonella enterica* serotype Typhimurium [10,11]. A3-APO was even more effective than conventional antibiotics administered as positive controls [12].

This study investigated the efficiency of oncocin, which has recently been developed by our laboratory to treat systemic *E. coli*, *E. cloacae*, *K. pneumoniae*, *P. aeruginosa* and *A. baumannii* infections [13]. The minimum inhibitory concentrations (MICs) of this 19mer sequence ranged from 0.25 μg/mL to 4 μg/mL for a panel of 32 bacterial strains. For the purpose of this investigation, the stability of oncocin against serum proteases using mouse serum, which in our experience degrades peptides equally as fast as human serum, was optimised. The half-life of oncocin in full mouse serum was increased from only 25 min to >8 h. The new peptide derivatives did not show any cytotoxic effects on human cell lines and were not haemolytic. Importantly, they were equally as active, or even more active, than oncocin.

2. Material and methods

2.1. Peptide synthesis

Peptides were synthesised on solid phase and were purified by reverse-phase high-performance liquid chromatography (RP-HPLC) as described previously [13]. α-Amino-3-guanidinopropionic acid (Agp) (Iris Biotech, Marktredwitz, Germany), nitro-arginine (Nir), *N*-methyl-arginine (Nmr), homoarginine (Har) (all from Bachem, Weil am Rhein, Germany) and β-homoarginine (βHr) (Fluka, Buchs, Switzerland) were coupled manually with diisopropylcarbodiimide/1-hydroxybenzotriazole (DIC/HOBt) activation. Side chains of trifunctional amino acids were protected with the following: 2,2,4,5,7-pentamethyldihydrobenzofuran-5-sulfonyl for arginine (Arg) and D-arginine (D-Arg); 2,2,5,7,8-pentamethylchroman-6-sufonyl for Har and βHr; 4-methoxy-2,3,6-trimethylphenyl-sulfonyl for Nmr; *tert*-butyl for aspartic acid (Asp) and tyrosine (Tyr); trityl for asparagine (Asn) and histidine (His); and *tert*-butyloxycarbonyl for Agp, lysine (Lys) and ornithine (Orn). For a peptidyl propylamide, Arg was coupled to 4-sulfamylbutyryl AM resin (Novabiochem®; Merck KGAa, Darmstadt, Germany) with benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) in the presence of *N*,*N*-diisopropylethylamine (DIPEA) at –20 °C. The peptide–acylsulphonamide resins were activated first with iodoacetonitrile (67 eq) and DIPEA (13 eq) in *N*,*N*-dimethylformamide (DMF) and were then cleaved with propylamine (50 eq) in DMF to yield the peptide propylamide [14].

2.2. Antibacterial activity

The non-pathogenic bacterial strains *E. coli* BL21AI and *Micrococcus luteus* ATCC 10240 were cultured in nutrient broth (Carl Roth GmbH, Karlsruhe, Germany), and pathogenic *E. coli* DSM 1103 and *K. pneumoniae* DSM 681 were grown overnight on nutrient agar (Carl Roth GmbH) at 37 °C. MIC values were determined in triplicate by a liquid broth microdilution assay in sterile 96-well plates (Greiner Bio-One GmbH, Frickenhausen, Germany) using a total volume of 100 μL/well. Aqueous peptide solutions (1 mg/mL) were serially two-fold diluted in 1% tryptic soy broth (TSB) starting at a concentration of 128 μg/mL and typically reducing to 0.5 μg/mL in eight steps. Overnight bacterial cultures were diluted with 1% TSB to 1.5×10^7 cells/mL (*E. coli* BL21AI), 4×10^7 cells/mL (*M. luteus* ATCC 10240) or 5×10^5 cells/mL (*E. coli* DSM 1103 and *K. pneumoniae* DSM 681). Then, 50 μL of these solutions was added to each well. The plates were incubated at 37 °C and the absorbance of each well was measured at 595 nm after 20 ± 2 h. The MIC was defined as the lowest peptide concentration where the absorbance value did not exceed that of the medium only.

2.3. Time–kill assay

Time–kill assays were performed for *E. coli* BL21AI in sterile polypropylene tubes containing a final volume of 2 mL of 1% TSB. The peptide concentrations were four-fold the corresponding MIC. The positive control did not contain any antibiotic. The inocula to be tested were prepared by adjusting the turbidity of an actively growing broth culture in 1% TSB to 1×10^7 colony-forming units (CFU)/mL. Tubes were continuously shaken on an orbital incubator at 37 °C and aliquots of 1 μL were taken in triplicate after 0, 1, 2, 4, 6 and 24 h. These aliquots were then spread with an inoculating loop directly, or after appropriate dilution, in triplicate onto an agarose plate containing 1% TSB. Colonies were counted after an incubation period of 24 h at 37 °C.

2.4. Serum stability

Peptides (7.5 μg) were dissolved in 25% (v/v) aqueous or undiluted pooled mouse serum (PAA Laboratories GmbH, Pasching, Austria) (100 μL) and were incubated at 37 °C [7]. Aliquots taken in duplicate or triplicate after 0, 30, 60, 120 and 240 min (and also 480 min for peptide O24) were precipitated by addition of trichloroacetic acid to a final concentration of 3% (v/v). After 10 min on ice, the samples were centrifuged and the supernatant was neutralised with sodium hydroxide solution (1 mol/L) and stored at −20 °C. Samples were analysed on an analytical Jupiter C_{18} column using a linear aqueous acetonitrile or methanol gradient containing 0.1% (v/v) trifluoroacetic acid. Metabolites were identified by matrix-assisted laser desorption/ionisation–time-of-flight mass spectrometry (MALDI-TOF-MS).

2.5. Haemolytic activity

Peptides were serially diluted from 600 μg/mL to 5 μg/mL in phosphate-buffered saline (PBS) (GIBCO, Invitrogen, Darmstadt, Germany) in a V-bottomed, 96-well polypropylene plate (Greiner Bio-One GmbH) to a final volume of 50 μL. Concentrated human erythrocytes were washed and suspended in PBS to a final concentration of 2%. Aliquots of this suspension (50 μL) were added to the peptide solution in each well and were incubated at 37 °C for 1 h. After centrifugation (1000 × *g*), the absorbance of the supernatants was determined in a fresh, flat-bottomed, 384-well plate (Greiner Bio-One GmbH) at 405 nm in a Paradigm™ microplate reader (Beckman Coulter, Wals, Austria).

2.6. Cytotoxicity

HeLa cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM)/Ham's F-12 medium with 10% (v/v) fetal bovine serum containing 1% (w/v) streptomycin, penicillin and neomycin. Then, $1–1.5 \times 10^4$ cells were seeded in the same medium into 96-well plates and were incubated overnight (37 °C, 5% CO_2). Subsequently, the peptide solutions (600 μg/mL) were added (100 μL/well) and