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Anti-infective efficacy of the lactoferrin-derived antimicrobial peptide HLR1r

Camilla Björn[a,b,c], Margit Mahlapuu[a,c], Inger Mattsby-Baltzer[d], Joakim Håkansson[a,b,*]

[a] Pergamum AB, Karolinska Institutet Science Park, Fogdevreten 2, SE-171 65 Solna, Sweden
[b] SP Technical Research Institute of Sweden, Medical Device Technology, Box 857, SE-501 15 Borås, Sweden
[c] The Lundberg Laboratory for Diabetes Research, Department of Molecular and Clinical Medicine, The Sahlgrenska Academy at University of Gothenburg, Blå stråket 5, SE-413 45 Gothenburg, Sweden
[d] Department of Infectious Diseases, Institute of Biomedicine, The Sahlgrenska Academy at University of Gothenburg, Box 440, SE-405 30 Gothenburg, Sweden

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ABSTRACT

Antimicrobial peptides (AMPs) have emerged as a new class of drug candidates for the treatment of infectious diseases. Here we describe a novel AMP, HLR1r, which is structurally derived from the human milk protein lactoferrin and demonstrates a broad spectrum microbicidal action in vitro. The minimum concentration of HLR1r needed for killing ≥99% of microorganisms in vitro, was in the range of 3–50 μg/ml for common Gram-negative and Gram-positive bacteria, including methicillin-resistant *Staphylococcus aureus* (MRSA), and for the yeast *Candida albicans*, when assessed in diluted brain-heart infusion medium. We found that HLR1r also possesses anti-inflammatory properties as evidenced by inhibition of tumor necrosis factor alpha (TNF-α) secretion from human monocyte-derived macrophages and by repression of interleukin-6 (IL-6) and plasminogen activator inhibitor-1 (PAI-1) secretion from human mesothelial cells, without any cytotoxic effect observed at the concentration range tested (up to 400 μg/ml). HLR1r demonstrated pronounced anti-infectious effect in in vivo experimental models of cutaneous candidiasis in mice and of excision wounds infected with MRSA in rats as well as in an ex vivo model of pig skin infected with *S. aureus*. In conclusion, HLR1r may constitute a new therapeutic alternative for local treatment of skin infections.

1. Introduction

The treatment of infectious diseases has recently been compromised due to emerging increase of bacterial resistance towards conventional antibiotics. Skin and soft tissue infections (SSTIs) including infected traumatic wounds, surgery wounds and superficial burn injuries is one therapeutic area where steep increase in resistance development over the past decades has seriously complicated the treatment outcome. The predominant causative bacterium in SSTIs across all continents is the Gram-positive *Staphylococcus aureus* [1]. Methicillin-resistant *S. aureus* (MRSA) strains, which are no longer susceptible to many broadly used antibiotics, are rapidly increasing in all geographic regions. According to recent studies, 45–90% of *S. aureus* isolates are resistant to methicillin [2–5]. Importantly, Gram-negative bacteria, such as *Pseudomonas aeruginosa* and *Escherichia coli*, are also frequently isolated from SSTIs and the alarming increase in drug-resistance and multidrug-resistance among the Gram-negative strains is a matter of great concern [1,4,6,7]. Over the last decade, a rapidly increasing incidence of polymicrobial infections often involving both Gram-positive and Gram-negative organisms has been reported [8]. In addition to bacteria, fungal pathogens such as *Candida* species are frequently causing infections particularly in burn wounds [9].

Dramatic increase in resistance towards conventional antibiotics and variation in causative microbes of wound infections emphasize the importance to identify novel, more potent antimicrobial therapies, which are effective against resistant strains and exhibit a broad action spectrum. Recently, antimicrobial peptides (AMPs) have emerged as a potential new strategy for prevention and treatment of bacterial infections [10,11]. These short peptides, generally containing between 10 and 50 amino acids, are important components of the innate immune system providing a first line of host defense against infections [12–14]. AMPs are produced in large quantities in response to microbial insult and they are effective against a broad range of microbes

* Corresponding author at: SP Technical Research Institute of Sweden, Medical Device Technology, Box 857, SE-501 15 Borås, Sweden.
E-mail address: joakim.hakansson@sp.se (J. Håkansson).

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[15]. In addition to antimicrobial action, a number of AMPs display immunomodulatory properties, which may provide further advantages in different therapeutic areas such as wound healing [12,15,16]. Importantly, no widespread resistance development toward AMPs has been reported, which relates to the AMPs mode-of-action involving attacking multiple low-affinity targets rather than one defined, high-affinity target characteristic for conventional antibiotics, which makes it difficult for target bacteria to defend themselves by a single resistance mechanism [16–18]. In particular, given that the bacterial cell membrane is presumed to be the main target of AMPs, it is challenging for bacteria to preserve the functional and structural integrity of the cell membrane and simultaneously avoid the membrane-disrupting activity of AMPs [17]. Although several AMPs have shown antimicrobial efficacy in both non-clinical and clinical studies, very few of these peptides have yet reached the market [19,12].

Here we describe a novel synthetic AMP, HLR1r, structurally derived from human lactoferrin, a protein present in exocrine secretions, particularly in milk, as well as in secondary granules in neutrophils [20,21]. Lactoferrin and lactoferrin-derived peptides have shown both antimicrobial and immunomodulatory activities in vitro as well as in experimental animal models [22–33]. HLR1r is identical to positions 21–32 of human lactoferrin, and similar peptide sequences have previously been shown to exhibit pronounced microbicidal effect in vitro [24,28]. This native sequence was further modified by adding a hexapeptide of Ser-Arg-Arg-Arg-Arg-Gly to the C-terminal end. It is widely accepted that cationicity is primarily responsible for the initial interaction of the AMP with the negatively charged membrane surface of the bacteria [13], and the Arg-rich motif was added to potentially facilitate this interaction. Moreover, both the N-terminal and the C-terminal ends of the peptide have been capped, i.e. the free NH_2- and –COOH groups have been converted into CH_3CONH- (i.e. AcNH-) and $-CONH_2$, respectively. This change makes the peptide ends neutral; furthermore, capping in the N-terminus resembles the corresponding residue in the native protein, while capping of the C-terminus could possibly enhance binding via the basic site to surface associated structures such as glycosaminoglycans [34].

HLR1r was chemically synthesized and screened for in vitro microbicidal activity against selected microbial strains in four different assay media. HLR1r was further evaluated for anti-inflammatory properties as measured by inhibition of secretion of tumor necrosis factor alpha (TNF-α) from lipopolysaccharide (LPS)-stimulated macrophages derived from the monocytic cell line THP-1 and production of interleukin-6 (IL-6) and plasminogen activator inhibitor-1 (PAI-1) from interleukin-1β (IL-1β)-stimulated mesothelial MeT-5A cells, as well as for its cytotoxicity toward the same cells. HLR1r was also assessed for its ability to reduce microbial counts in three different infected wound models: an in vivo yeast skin infection model in mice, an ex vivo pig skin infection model and an in vivo excision wound infection model in rats.

2. Materials and methods

2.1. Peptide and antibiotics

HLR1r peptide (Ac-Phe-Gln-Trp-Gln-Arg-Asn-Met-Arg-Lys-Val-Arg-Gly-Ser-Arg-Arg-Arg-Arg-Gly-NH_2) was produced using Fmoc solid phase technology at the Department of Organic Chemistry, University of Gothenburg, Gothenburg, Sweden (yeast skin infection model) or at Bachem AG, Bubendorf, Switzerland (all other experiments). The peptide was identified by electrospray ionization mass spectrometry and the purity of the peptide was assessed by HPLC. Mupirocin (Bactroban, 2% ointment, GlaxoSmithKline, Brentford, UK) was used as control antibiotic.

2.2. In vitro minimum microbicidal concentration (MMC) assay

The microbicidal effect of HLR1r was assessed against *S. aureus* (ATCC 12600; American Type Culture Collection, Manassas, VA, USA), MRSA (ATCC 33591), *Streptococcus pyogenes* (ATCC 12344), *P. aeruginosa* (ATCC 15442), *E. coli* (CCUG 31246; Culture Collection, University of Gothenburg, Sweden), *Propionibacterium acnes* (ATCC 6919), and *Candida albicans* (ATCC 64549) using a MMC assay as described previously in Ref. [24,28,35,36]. The MMC assay was performed in 100x diluted brain-heart infusion broth (BHI_{dil}) or in 2x diluted heat-inactivated simulated wound fluid (SWF_{dil}). BHI_{dil} has been previously used to assess microbicidal activity of lactoferrin-derived peptides under low-ionic strength conditions to increase the level of sensitivity [24,27,28] and SWF_{dil} was selected to create an assay medium mimicking the clinical conditions in the wound bed.

In brief, all microorganisms, except *P. acnes*, were cultured in BHI broth (3.7%; Difco, BD Diagnostics, Franklin Lakes, NJ, USA) overnight on a shaker at 37 °C and 250 rpm. The cultures were thereafter diluted 1:10 in fresh BHI broth and incubated for an additional 2 h to reach exponential growth phase. The microorganisms were thereafter centrifuged at 900*g* and re-suspended in BHI_{dil} to a density of 10^7 CFU/ml, as estimated by optical density measurements at 600 nm. *P. acnes* were cultured on blood agar plates under anaerobic conditions, i.e. in an anaerobic jar containing oxygen absorber (Oxoid Ltd., Basingstoke, UK) at 37 °C for 5 days. The bacteria were thereafter harvested in BHI broth, centrifuged at 900*g* and re-suspended in BHI broth to a density of 10^7 CFU/ml. The density of the microbial suspension was confirmed by viable count estimation on blood agar plates for all the strains used.

Starting with 200 μg/ml as the highest concentration (400 μg/ml for *P. acnes*), HLR1r was serially diluted in duplicate by twofold steps in BHI_{dil} (unsupplemented or supplemented with either 85 mM or 150 mM NaCl) or in SWF_{dil}, where SWF is a 1:1 mixture of 0.1% peptone water (Oxoid Ltd.) in 150 mM NaCl and fetal bovine serum (FBS; PAA Laboratories GmbH, Pasching, Austria). A volume of 100 μl of the peptide dilutions were added to the wells of a 96-well round bottom polypropylene plate (Nunc, Roskilde, Denmark) and mixed with 5 μl of the microbial suspension, reaching a final density of approximately 5×10^5 CFU/ml. The microtiterplate was incubated at 37 °C for 2 h. A volume of 5 μl was withdrawn from each well and added as drops onto agar plates (Columbia agar; Oxoid Ltd.) supplemented with 5% defibrinated horse blood (Swedish National Veterinary Institute, Uppsala, Sweden). The blood agar plates were incubated at 37 °C overnight, except for *P. acnes*, in which case the agar plates were incubated under anaerobic conditions at 37 °C for 3 days. Viable cell count was performed in each spot formed by the drops (three from each well) and from the mean value, the final density of microorganisms in the well was calculated and compared with the initial inoculum. Maximum 50 individual colonies could be counted per spot, hence only a killing activity of ≥98% could be detected. Less than 10 CFU/spot (corresponding to <2000 CFU/ml in the well) was defined as the MMC_{99} value, killing >99.6% of the initial inoculum. A difference in MMC_{99} values by a factor of four (i.e. two titer steps) was regarded as significantly different.

2.3. In vitro anti-inflammatory assay using macrophages

The in vitro anti-inflammatory efficacy of HLR1r was evaluated using monocyte-derived macrophages as previously described in Ref. [31]. In brief, the THP-l cell line (ATCC TIB-202) corresponding to human monocytes was maintained in RPMI 1640 (PAA Laboratories GmbH) supplemented with 10% FBS (PAA), 1 mM sodium pyruvate (Sigma-Aldrich, St. Louis, MO, USA), and 20 mM HEPES (PAA). The cell density was adjusted to 10^6 cells/ml and