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Chicken cathelicidin-2-derived peptides with enhanced immunomodulatory and antibacterial activities against biological warfare agents

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ABSTRACT

Host defence peptides (HDPs) are considered to be excellent candidates for the development of novel therapeutic agents. Recently, it was demonstrated that the peptide C1-15, an *N*-terminal segment of chicken HDP cathelicidin-2, exhibits potent antibacterial activity while lacking cytotoxicity towards eukaryotic cells. In the present study, we report that C1-15 is active against bacteria such as *Bacillus anthracis* and *Yersinia pestis* that may potentially be used by bioterrorists. Substitution of single and multiple phenylalanine (Phe) residues to tryptophan (Trp) in C1-15 resulted in variants with improved antibacterial activity against *B. anthracis* and *Y. pestis* as well as decreased salt sensitivity. In addition, these peptides exhibited enhanced neutralisation of lipopolysaccharide (LPS)-induced release of pro-inflammatory cytokines in human peripheral blood mononuclear cells (PBMCs). The antibacterial and LPS-neutralising activities of these C1-15-derived peptides are exerted at concentrations far below the concentrations that are toxic to human PBMCs. Taken together, we show that Phe→Trp substitutions in C1-15 variants enhances the antibacterial and LPS-neutralising activities against pathogenic bacteria, including those that may potentially be used as biological warfare agents.

1. Introduction

Exposure to biological warfare agents may cause a highly progressive, acute infection that may be lethal in some forms. In these cases, use of broad-spectrum antibiotics might be life saving. In addition, the number of antibiotic-resistant microorganisms is increasing, underlining the growing need for novel antimicrobial compounds.

Host defence peptides (HDPs) are excellent candidates for the development of novel broad-spectrum antibiotics. In general, HDPs exhibit potent broad-spectrum antibacterial and/or immunomodulatory properties. Moreover, HDPs have a low tendency to select resistant strains [1,2].

We previously demonstrated that the *N*-terminal fragment of the HDP chicken cathelicidin-2 (CATH-2), namely the peptide C1-15, possesses broad-spectrum antibacterial activity while being non-haemolytic against erythrocytes and is therefore a promising lead for the development of novel antimicrobial drugs with broad-spectrum activity [3].

In this study, we explored the use of C1-15 as a potential novel lead compound to combat biowarfare agents. In doing so, we aimed at broadening the antibacterial activity of C1-15 by substitution of phenylalanine (Phe) residues on the non-polar face by tryptophan (Trp) residues. The antibacterial activity and toxicity against human peripheral blood mononuclear cells (PBMCs) of C1-15 variants were evaluated. In addition, the anti-inflammatory activity of C1-15 variants was examined.

2. Material and methods

2.1. Peptide synthesis

C1-15 (RFGRFLRKIRRFRPK) and the C1-15 variants F2W (RWGRFLRKIRRFRPK), F5W (RFGRWLRKIRRFRPK), F12W (RFGRFLRKIRRWRPK) and F2,5,12W (RWGRWLRKIRRWRPK) were synthesised by Fmoc chemistry using a SYRO peptide synthesiser (MultiSynTech, Bochum, Germany) as described by Bikker et al. [4].

2.2. Antibacterial activity

The antimicrobial activity of C1-15 variants was evaluated against Gram-positive *Bacillus anthracis* Vollum strain ATCC 14578 (American Type Culture Collection, Manassas, VA) and methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 43300 as well as against the Gram-negative virulent *Yersinia pestis* NCTC 08775 (National Collection of Type Cultures, London, UK) and *Vibrio cholerae* (clinical isolate, Slotervaart Hospital, Amsterdam, The Netherlands). All bacteria were grown and maintained in tryptic soy broth (TSB) (bioTRADING, Mijdrecht, The Netherlands) under aerobic conditions at 35 °C (*B. anthracis*, MRSA and *V. cholera*) or 26 °C (*Y. pestis*). The antimicrobial activity of C1-15 and C1-15 variants was determined using colony counting assays as described previously [3].

2.3. Isolation and culture of peripheral blood mononuclear cells

Human PBMCs were isolated from buffy coats (Sanquin, Rotterdam, The Netherlands) as reported previously [3] and were seeded at 0.5×10^6 cells/well into a Corning Costar 96-well flat-bottom culture plate (Corning Inc., Corning, NY) in RPMI-1640 (Lonza BioWhittaker, Basel, Switzerland) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (FBS Gold; PAA, Pasching, Austria), 100 U/mL penicillin and 100 µg/mL streptomycin (Lonza BioWhittaker).

2.4. Cytotoxicity assay

The toxic effect of C1-15 variants on PBMCs was evaluated using WST reagents (Roche Diagnostics, Mannheim, Germany). Cells were incubated for 24 h with culture medium and C1-15 variants at 37 °C and 5% CO₂. Following centrifugation, supernatants were removed and 100 µL of fresh RPMI-1640 supplemented with 10% FBS and 10 µL of WST-1 reagents was added and the cells were further incubated. After 60 min incubation time, absorbance was determined at 450 nm, with a reference wavelength at 650 nm.

2.5. Neutralisation of the lipopolysaccharide (LPS)-induced cytokine response by C1-15 variants

PBMCs were used to study the modulation of LPS-induced cytokine secretion by C1-15 variants. PBMCs were stimulated with 1 ng/mL ultrapure LPS from *Escherichia coli* O111:B4 (InvivoGen, San Diego, CA) for 24 h in the absence or presence of various

concentrations of C1-15 variants. Following incubation, cell culture supernatants were collected and stored at -20°C until further analysis.

2.6. Cytokine measurements

Levels of interleukin (IL)-6 were determined by enzyme-linked immunosorbent assay (ELISA) using the commercial PeliKine Compact™ Human ELISA Kit (Sanquin, Amsterdam, The Netherlands) following the manufacturer's recommendations.

2.7. LPS binding by C1-15 variants

C1-15 variants were examined for LPS-neutralising activity in a limulus amoebocyte lysate (LAL) assay. Briefly, 25 μL of peptide at concentrations from 0 to 20 μM diluted in RPMI-1640 (Lonza BioWhittaker) were incubated with 25 μL of a 2 ng/mL ultrapure LPS from *E. coli* O111:B4 (InvivoGen) for 60 min at 37°C in a 96-well endotoxin-free plate (Hycult Biotechnology, Uden, The Netherlands). Residual LPS activity was detected using the LAL assay according to the manufacturer's recommendations (Hycult Biotechnology).

2.8. Statistical analysis

Statistical significance was determined by two-way analysis of variance (ANOVA) followed by post hoc testing by the Bonferroni method. A *P*-value of <0.05 was considered statistically significant. Values shown are expressed as mean $\pm$ standard error of the mean.

3. Results

3.1. Antibacterial activity of C1-15 and C1-15 variants

The *N*-terminal segment of CATH-2, peptide C1-15, affected the survival of all tested bacteria (Fig. 1)

To enhance the antimicrobial activity of C1-15, a panel of C1-15 variants with Phe→Trp substitutions was designed. In comparison with Phe, Trp contains a more bulky hydrophobic side chain consisting of an indole chain. Since there are three Phe residues on the non-polar face of C1-15, variants were synthesised with Phe→Trp substitutions at positions 2 (F2W), 5 (F5W) and 12 (F12W). In addition, a triple Phe→Trp variant was synthesised (F2,5,12W).

Compared with C1-15, a single Phe→Trp substitution (F2W, F5W and F12W) improved the antibacterial activity against *Y. pestis* and *B. anthracis*. Survival of *Y. pestis* was reduced with 50% by 5 μ M F2W, F5W or F12W compared with 20 μ M for C1-15 (Fig. 1B). Moreover, 2 μ M of F2W, F5W or F12W led to a 50% decline in *B. anthracis* survival compared with 4 μ M for C1-15 (Fig. 1A,D. Antibacterial activity against *Y. pestis* and *B. anthracis* was further improved by multiple substitutions, with 2.5 μ M and 1 μ M, respectively, of F2,5,12W needed to reduce the survival of the bacteria by 50% (Fig. 1A,D.

3.2. Antibacterial activity in the presence of salts

The antibacterial activity of CATH-2 against MRSA was not affected by 100 mM NaCl (data not shown). However, C1-15 lost its antibacterial activity in the presence of 100 mM NaCl. Even the highest peptide concentration used (40 μ M) could only reduce the survival by 1 log unit. However, Trp substitutions resulting in F2,5,12W partly restored the antibacterial activity in the presence of NaCl (Fig. 2).

3.3. Cytotoxicity of C1-15 variants

C1-15 variants were evaluated for their toxicity to human PBMCs with and without the presence of 10% FBS. Cytotoxicity of C1-15 to PBMCs in the presence of FBS was negligible. Substitution of Phe by Trp residues increased the cytotoxicity to PBMCs up to 75% at 40 μ M after multiple substitutions (F2,5,12W) (Fig. 3). Similar experiments in the absence of FBS showed that FBS only had a marginal protective effect against peptide cytotoxicity (data not shown).

3.4. Neutralisation of the LPS-induced cytokine response

PBMCs were stimulated with LPS in the presence of various concentrations of C1-15 variants. The concentration of the pro-inflammatory mediator IL-6 was measured in culture supernatants (Fig. 4A).

Substitution of Phe residues in C1-15 by Trp residues improved the LPS-neutralising capacity by improving inhibition of the LPS-induced release of IL-6. Multiple Phe→Trp

substitutions in C1-15 resulted in even higher LPS-neutralising activity; 10 μ M of F2,5,12W significantly inhibited 85% of the IL-6 LPS response (Fig. 4A).

3.5. LPS binding by C1-15 variants

To verify whether the improved LPS-neutralising activities of C1-15 variants could be contributed to improved LPS binding, C1-15 variants were evaluated for their ability to bind LPS in a LAL assay. Phe→Trp substitution in C1-15 enhanced the LPS-binding capacity of the peptides (Fig. 4B).

4. Discussion

During military operations or bioterrorist attacks, immediate diagnosis or identification of the bacterial agent is not always possible. In case of exposure from an unknown source, the availability and usability of broad-spectrum antimicrobials can be vital. Furthermore, owing to the use and misuse of current antimicrobials, growing numbers of multiresistant bacteria have arisen, including those that might potentially be used for offensive purposes, implying that there is a need for new broad-spectrum antimicrobials.

Recently, it was reported that the HDP C1-15, a truncated variant of CATH-2, possesses strong broad-spectrum antibacterial activity. In addition, this peptide is non-haemolytic against erythrocytes [3]. In this study, we extended the earlier reported broad antibacterial activity with more bacteria, including bacteria often related to bioterrorist

attacks (*B. anthracis*, *V. cholera* and *Y. pestis*). In addition, we aimed to enhance the antibacterial activity of C1-15 by substituting Phe residues by Trp residues.

Substitutions of C1-15 Phe to Trp residues resulted in a peptide containing a large number of Arg and Trp residues. Previously it was reported by others that Arg and Trp complement each other well for the purpose of antimicrobial activity [5]. The cationic charge of Arg provides an effective means of attraction of the peptides to the target membranes, and hydrogen bonding facilitates the interaction with the negatively charged surfaces of bacterial membranes. The hydrophobic bulk of Trp residues play a role in membrane association because it preferentially interacts with the interfacial region of membranes [6]. In combination, Arg and Trp residues are capable of participating in stronger cation- π interactions compared with Arg and Phe residues [7]. Such interactions would facilitate deeper embedding of the peptides in the membrane and disrupt the membrane structure [5].

In line with these data, we observed that single Phe substitutions (F2W, F5W and F12W) augmented the antibacterial activity by a factor of four against *Y. pestis* and by a factor of two against *B. anthracis*. Multiple substitutions (F2,5,12W) further enhanced antibacterial activity: the peptide was eight and four times more potent compared with C1-15 in reducing the survival of *Y. pestis* and *B. anthracis*, respectively.

The ability to resist salt is highly relevant for HDPs in order to function under physiological conditions. As reported earlier by Xiao et al. [8] and confirmed by us,

CATH-2 is resistant to salt, but C1-15 almost completely lost its antibacterial activity in the presence of 100 mM NaCl. However, we could restore the antibacterial activity in the presence of salt by Phe to Trp substitutions.

Overall, these findings show that the insertion of Trp residues can make peptides less salt-sensitive and more potent in bacterial killing.

Bacterial killing by antimicrobial compounds may cause a release of endotoxin (LPS) from Gram-negative bacteria, which subsequently might lead to an exaggerated immune response. This can result in early sepsis, in which high levels of cytokines and inflammatory mediators become destructive, causing organ failure, cardiovascular shock and even resulting in death [9]. Some HDPs are found to counteract the development of septic shock by neutralising endotoxin [10]. Although CATH-2 is able to inhibit the LPS-induced pro-inflammatory cytokine responses, the *N*-terminal part, C1-15, showed only moderate LPS-neutralising activities [3]. Phe→Trp substitutions in C1-15, resulting in peptide F2,5,12W, significantly increased the LPS-neutralising capacity. Recently, we [11] and others [12] reported the importance of hydrophobicity in neutralising LPS cytokine responses. In line with our observations, Nan et al. [12] showed that substitution of Trp by Lys residues in the Trp-rich peptide indolicidin resulted in decreased hydrophobicity and loss of LPS-neutralising activity. Previously it was shown that inhibition of the pro-inflammatory response is mainly established by direct binding of cationic HDPs to LPS [11,13]. In line with these observations, we found a correlation between LPS-neutralising activities and LPS binding.

It has been demonstrated that high concentrations of cationic HDPs containing a high amount of Trp residues may be toxic to the host [14]. In the present study, we observed that an increased number of Trp residues results in increased cytotoxicity against mammalian cells such as PBMCs. However, the Trp substitutes exerted broad antibacterial and LPS-neutralising activities at much lower concentrations than their toxic effects on human PBMCs.

In summary, we successfully increased the antibacterial activity of C1-15 and made it less salt sensitive by replacement of Phe by Trp residues. In addition, F2,5,12W showed good neutralising activities of LPS cytokine responses.

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Competing interests

None declared.

Ethical approval

Not required.

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Fig. 1. Bactericidal activity of C1-15 (○), F2W (■), F5W (●), F12W (▼) and F2,5,12W (×) against (A) *Vibrio cholera*, (B) *Yersinia pestis*, (C) methicillin-resistant *Staphylococcus aureus* (MRSA) and (D) *Bacillus anthracis*. Each point represents the mean + standard error of the mean of at least four experiments. * Significantly different compared with C1-15 ($P < 0.05$). CFU, colony-forming units.

Fig. 2. Salt sensitivity of C1-15 and F2,5,12W: bactericidal activity against methicillin-resistant *Staphylococcus aureus* (MRSA) of C1-15 (○) and F2,5,12W (×) without salt and of C1-15 (●) and F2,5,12W (*) in the presence of 100 mM NaCl. CFU, colony-forming units.

Fig. 3. Cytotoxicity of C1-15 (○), F2W (■), F5W (●), F12W (▼) and F2,5,12W (×) on human peripheral blood mononuclear cells. Each point represents the mean + standard error of the mean of three different donors. * Significantly different compared with C1-15 ($P < 0.05$).

Fig. 4. (A) Comparison of the lipopolysaccharide (LPS)-neutralising activity of C1-15 (○), F2W (■), F5W (●), F12W (▼) and F2,5,12W (×) determined by inhibition of LPS-induced interleukin (IL)-6 release. The amount of IL-6 resulting from cells incubated with LPS alone was set to 100% (52.4 ± 5.4 ng/mL). (B) Limulus amoebocyte lysate assay. Each point represents the mean + standard error of the mean of three independent experiments. * Significantly different compared with C1-15 ($P < 0.05$).