Design of Short Indolicidin Analogs with Enhanced Prokaryotic Selectivity

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Indolicidin (ID) is a 13-residue Trp-rich antimicrobial peptide (AMP) isolated from bovine neutrophils. In addition to having a high antimicrobial potency, it is also toxic to mammalian cells. To develop novel ID-derived AMPs with shorter lengths and enhanced prokaryotic selectivities (meaning potent antimicrobial activity against bacterial cells without toxicity against mammalian cells) over the parental ID, several ID analogs were designed and synthesized. Finally, 10-residue ID analogs (SI, SI-PA, SI-WF and SI-WL) with much higher prokaryotic selectivity than the parental ID were developed. Our results suggest that the hydrophobic and aromatic amino acids at the central position of the analog SI with the highest prokaryotic selectivity are important for potent antimicrobial activity, but two Pro residues do not affect antimicrobial activity. The order of prokaryotic selectivity for ID and its designed analogs was SI > SI-PA > SI-WF > SI-WL > ID > SI-WA. Taken together, our designed short ID analogs could be developed as therapeutic agents for treating bacterial infections.

Key words: antimicrobial peptide, indolicidin, short analogs, prokaryotic selectivity

Introduction

Antimicrobial peptides (AMPs) represent an important component of innate immunity and host defenses against infectious agents [2, 14]. AMPs have recently gained much attention because of their ability to overcome such resistance and have emerged as a potential new class of antibacterial agents with new modes of actions [2, 14].

Indolicidin (ID) (ILPWKWPWPWR-NH₂), a member of the cathelicidin family, is a 13-residue cationic AMP isolated from the cytoplasmic granules of bovine neutrophils [13]. ID is one of the smallest natural linear cationic AMPs known to date. Its unique composition distinguishes it from the α-helical and β-structures cationic peptides. It has a unique composition consisting of 39% tryptophan and 23% proline, and the native peptide is amidated at the C-terminus [13]. ID adopts an extended wedge-type conformation in the presence of biological membranes, with the hydrophobic Trp residues in the trough flanked by positively charged regions [6, 10].

It exhibits significant activity against a wide range of targets, including bacteria, fungi, protozoa and human immunodeficiency virus (HIV-1). Owing to its relatively short length and broad spectrum of antimicrobial activity, ID is a promising candidate for the development of antimicrobial drugs [5, 11, 12]. However, toxicity towards eukaryotic cells (typically measured as hemolytic activity toward human red blood cells) prevents its successful clinical application. Thus, considerable efforts have been made to decrease toxicity of ID against mammalian cells [1, 8, 9].

In this study, to develop novel ID analogs with shorter length and enhanced prokaryotic selectivity (means having potent antimicrobial activity against bacterial cells without toxicity against mammalian cells) compared to parental ID, several short ID analogs were designed and synthesized. The prokaryotic selectivity of the peptides was investigated by examining their antimicrobial activity against Gram-positive and Gram-negative bacterial strains and their hemolytic activity against human red blood cells.

Materials and Methods

Materials
Rink amide 4-methylbenzhydrylamine (MBHA) resin
and 9-fluorenylmethoxycarbonyl (Fmoc) amino acids were obtained from Calbiochem-Novabiochem (La Jolla, CA). Other reagents used for peptide synthesis included trifluoroacetic acid (TFA; Sigma), piperidine (Merck), dicyclohexylcarbodiimide (DCC; Fluka), N-hydroxybenzotriazole hydrate (HOBT; Aldrich) and dimethylformamide (DMF; peptide synthesis grade; Biolab). All other reagents were of analytical grade. The buffers were prepared in double glass-distilled water.

**Peptide synthesis**

ID and its short analogs listed in Table 1 were prepared by solid-phase peptide synthesis (SPPS) [7]. DCC and HOBT were used as coupling reagents, and 10-fold excess of Fmoc-amino acids was added during every coupling cycle. After cleavage and deprotection with a mixture of trifluoroacetic acid/H₂O/thioanisole/phenol/ethanedithiol/triisopropylsilane (81:5.5:5.5:2.5:1, v/v) for 2 h at room temperature, crude peptides were repeatedly extracted with diethyl ether and purified by reverse-phase high-performance liquid chromatography (RP-HPLC) on a preparative Vydac C₁₈ column (15 μm, 20 mm×250 mm) using an appropriate 0-90% water/acetonitrile gradient in the presence of 0.05% TFA. The final purity of the peptides (>98%) was assessed by RP-HPLC on an analytical Vydac C₁₈ column (4.6 mm×250 mm, 300 Å, 5-μm particle size). The observed mass of each peptides determined by matrix-assisted laser-desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS) (Shimadzu, Japan) were a good agreement with their calculated MS (Table 1).

**Antimicrobial activity (MIC)**

All bacterial strains were procured from the Korean Collection for Type Cultures (KCTC) at the Korea Research Institute of Bioscience and Biotechnology (KIRIBB). The antimicrobial activity of the peptides was examined by using the broth microdilution method in sterile 96-well plates. Aliquots (100 μl) of a bacterial suspension at 2×10⁶ colony-forming units (CFU)/ml in 1% peptone were added to 100 μl of the peptide solution (serial 2-fold dilutions in 1% peptone). After incubation for 18-20 h at 37°C, bacterial growth inhibition was determined by measuring the absorbance at 600 nm with a Microplate Autoreader EL 800 (Bio-Tek Instruments, VT). The minimal inhibitory concentration (MIC) was defined as the minimum peptide concentration inhibited bacteria growth.

**Hemolytic activity**

Fresh human red blood cells (hRBCs) were washed 3 times with PBS (35 mM phosphate buffer, 0.15 M NaCl, pH 7.4) by centrifugation for 7 min at 1000×g, and resuspended in PBS. The peptide solutions (serial 2-fold dilutions in PBS) were added to 100 μl of hRBC suspension [4% (v/v) in final] in PBS to a final volume of 200 μl, and incubated for 1 h at 37°C. Samples were centrifuged at 1000×g for 5 min, and hemoglobin release was monitored by measuring the supernatant absorbance at 405 nm with a Microplate ELISA Reader (Bio-Tek Instruments, VT, USA). hRBCs in PBS (Ablank) or 0.1% Triton X-100 (Atriton) were used as the negative and positive controls, respectively. The hemolysis percentage was calculated according to the equation:

% hemolysis = 100 × [(Asample - Ablank)/(Atriton - Ablank)]

**Results and Discussion**

**Design and Synthesis of Short Indolicidin (ID) analogs**

Firstly, we designed a symmetric 10-meric analog, SI (RRWPWWPPWR-NH₂) based on the sequence of ID. In SI-WF, SI-WL and SI-WA, 4 Trp residues of SI were substituted with Phe, Leu and Ala, respectively. In SI-PA,
Antimicrobial and Hemolytic Activities

We examined the antimicrobial activities of the peptides against a representative set of bacterial strains, including three Gram-negative bacteria (Escherichia coli [KCTC 1682], Pseudomonas aeruginosa [KCTC 1637] and Salmonella typhimurium [KCTC 1926]) and three Gram-positive bacteria (Staphylococcus epidermidis [KCTC 1917] and Staphylococcus aureus [KCTC 1621] and Bacillus subtilis [KCTC 3068]). The minimal inhibitory concentration (MIC) values of ID and its analogs against six different bacterial strains are shown in Table 2.

A symmetric 10-meric analog, SI, showed a 2-4-fold increased antimi crobial activity against Gram-negative bacteria compared to parental ID. In contrast, the antimicrobial activity of SI against Gram-positive bacteria was almost equivalent to that of ID. SI-WF displayed almost similar antimicrobial activity compared to SI. SI-WL was less 2-8-fold less active than SI, W→A substitution in SI (SI-A) induced a drastic decrease in antimicrobial activity. These results suggested that the hydrophobic aromatic amino acid in the central position of SI is important for its potent antimicrobial activity. Furthermore, the antimicrobial activity of SI-PA was almost equal to that of SI, indicating that two Pro residues of SI did not affect on antimicrobial activity.

The cytotoxicity of the peptides to mammalian cells of peptides by measuring their hemolytic activity toward human red blood cells (hRBCs) was measured. As shown in Fig. 1, ID induced 50% hemolysis at the concentration of 151 µg/ml, but all short ID analogs exhibited no or less hemolytic activity even at the highest concentration of 200 µg/ml.

Prokaryotic Selectivity

The therapeutic potential of peptide antimicrobial drugs lies in their prokaryotic selectivity to selectively kill bacterial cells without exhibiting significant cytotoxicity toward mammalian cells. To assess prokaryotic selectivity of the peptides, their therapeutic index (TI) was calculated as a measure of the relative safety of the drug [3, 4, 15]. The TI of the peptides was calculated as the ratio of the HC50 that produced 50% hemolysis against human red blood cells to the GM (geometric mean of MICs against six microorganisms) (Table 3). Larger values of TI correspond to greater prokaryotic selectivity. When there was signi-

<table>
<thead>
<tr>
<th>Peptides</th>
<th>Minimal Inhibitory Concentration (MIC) (µg/ml)</th>
<th>Gram-positive bacteria</th>
<th>GM (Geometric mean of MICs)</th>
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<td>Gram-negative bacteria</td>
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<td>E. coli</td>
<td>P. aeruginosa</td>
<td>S. typhimurium</td>
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<td>Indolicidin (ID)</td>
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<td>SI</td>
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<td>SI-PA</td>
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*The lowest peptide concentration that causes 100% inhibition of microbial growth.
²Geometric mean of MICs against 3 Gram-negative strains tested.
²²Geometric mean of MICs against 3 Gram-positive strains tested.
²²²Geometric mean of MICs against all of the 6 strains tested.
significant no hemolysis at the highest concentration tested (200 
μg/ml), 400 μg/ml was used for the TI calculation, since the test was carried out by two-fold serial dilution.

Except for SI-A, all short ID analogs displayed higher TI compared with parental ID because of their decreased hemolytic activity and increased antimicrobial activity. The order of prokaryotic selectivity for the peptides was SI>SI-PA>SI-WF>SI-WL>ID>SI-WA (Table 3).

In conclusion, novel 10-meric ID analogs (SI, SI-PA, SI-WF and SI-WL) with shorter length and enhanced prokaryotic selectivity compared to parental ID were developed. Therefore, our designed short ID analogs could be developed as therapeutic agent for treating bacterial infections.

REFERENCES

국문초록

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