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Structure-activity relationship of indolicidin, a Trp-rich antibacterial peptide

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A series of Trp and Arg analogs of antibacterial indolicidin (Ind) was synthesized and the antimicrobial and hemolytic activities were investigated. [L^9]Ind, [L^{11}]Ind, [K^8,L^9]Ind and [$K^{6,8}$,L^9]Ind showed desirable characteristics, exhibiting negligible hemolytic activity while keeping strong antibacterial activity. The results indicated that the Trp residue at position 11 essentially contributes to both activities and one can not be exchanged for the other, whereas the Trp residues at positions 4 and 9 play important roles in antimicrobial and hemolytic activities, respectively. The Trp residues at positions 6 and 8 play no important roles in biological activities. We then found that the retro analog of Ind showed higher antibacterial activity than Ind against both Gram-positive and Gram-negative bacteria but remarkably lower hemolytic activity than that of Ind.

Keywords: indolicidin; antimicrobial peptide; retro peptide; antibiotic activity; hemolytic activity

Introduction

Antibacterial peptides, which interact with bacterial cell membranes, have 10–40 amino acid residues and generally show their biological activities by membrane perturbation [1]. Such peptides, e.g. gramicidin S, β-defensins, magainins, maculatin 1.1, parasin I, and so on, are expected to be useful as medical supplies, because they quickly lyse bacterial membranes and avoid the appearance of resistant bacteria. Synthetic analogs, which improved the antimicrobial activities, were also developed to demonstrate their highly efficient action toward bacterial cells [2–12]. One of the most significant structural features of these antibiotic peptides is the existence of cationic amino acid residues, which perturb bacterial cell membranes by electrostatic interaction, leading to subsequent cell lysis by the interaction of other residues, such as hydrophobic and/or electrostatic interactions, with cell membranes.

Indolicidin (Ind), a cationic antibacterial tridecapeptide with unprotected amino and amidated carboxyl termini, was isolated from the cytoplasmic granules of bovine neutrophils [13]. Ind has antibacterial activity against a wide range of microorganisms, Gram-positive and Gram-negative bacteria [14–18]. It can be categorized in the cathelicidin family, Trp- and Arg-rich antimicrobial peptides, which are highly active even at short peptide length [19]. It is noticeable that Ind has five Trp residues in 13 sequence positions and two consecutive Arg residues at the C-terminus. The high ratio of Trp content in Ind is characteristic of the natural antimicrobial peptides, and is regarded as an essential feature for exhibiting high antibacterial activity, because these Trp residues have a natural liking for the interfacial region of lipid bilayers [20]. The association of cationic antibacterial peptides with cell membranes generally causes not only the lysis of bacteria but also hemolysis, so further work is needed to elucidate the action mechanism of Ind [21,22].

Several studies have been done to investigate the role of the residues and improve the activities of Ind by synthesizing analogous peptides [23–26]. Uchida *et al*. reported that the antibacterial activity of Arg-lacking analogs of Ind was negative, indicating that the strong cation is required for the activity [23]. Analogs with a single Trp at position 4, 8 or 11 and others replaced by Leu were found to reduce hemolytic activity with retaining antibacterial activity [24]. Analogs containing Trp-like unnatural amino acids such as 3-(2-naphthyl)-Ala were synthesized, resulting in enhanced activity of Ind [25]. Furthermore, modifications of Ind were made by substituting Pro with Ala, and by enhancing the overall charge [26,27]. The former modification led to conformational changes exhibiting increased activity against Gram-positive bacteria, whereas the latter improved activity against Gram-negative bacteria and, moreover, made the peptide less hemolytic than Ind [26,27]. Recently, Pro-rich short analogs were reported as antimicrobial peptides, which had low hemolytic activities [28]. The above studies indicated that (i) two Arg residues are essential for exhibiting enough antibacterial activity, (ii) Trp residues or large aromatic moieties play important but complex roles in acting as antimicrobial peptides, and (iii) some specific Trp analogs can exhibit lower hemolytic activities than Ind. However, no examination has been tried to obtain peptides having high antibacterial activity against both Gram-positive and Gram-negative bacteria but low hemolytic activity. Furthermore, light-sensitive Trp residues are preferable to be changed as much

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Table 1. Amino acid sequences and molecular weights of Ind and its analogs[a]

Name	Sequence	MS (m/e) Found	MS (m/e) Calcd (M + 1)
Ind	I-L-P-W-K-W-P-W-W-P-W-R-R-NH_2	1906.2	1907.3
[L^4]Ind	I-L-P-**L**-K-W-P-W-W-P-W-R-R-NH_2	1833.5	1834.2
[L^6]Ind	I-L-P-W-K-**L**-P-W-W-P-W-R-R-NH_2	1833.9	1834.2
[L^8]Ind	I-L-P-W-K-W-P-**L**-W-P-W-R-R-NH_2	1833.6	1834.2
[L^9]Ind	I-L-P-W-K-W-P-W-**L**-P-W-R-R-NH_2	1833.2	1834.2
[L^{11}]Ind	I-L-P-W-K-W-P-W-W-P-**L**-R-R-NH_2	1834.0	1834.2
[$L^{4,6,8,9,11}$]Ind	I-L-P-**L**-K-**L**-P-**L**-**L**-P-**L**-R-R-NH_2	1542.7	1542.0
[$F^{4,6,8,9,11}$]Ind	I-L-P-**F**-K-**F**-P-**F**-**F**-P-**F**-R-R-NH_2	1711.4	1712.1
[$Y^{4,6,8,9,11}$]Ind	I-L-P-**Y**-K-**Y**-P-**Y**-**Y**-P-**Y**-R-R-NH_2	1791.6	1792.0
[$L^{4,6}$]Ind	I-L-P-**L**-K-**L**-P-W-W-P-W-R-R-NH_2	1761.5	1761.2
[$K^{4,6}$]Ind	I-L-P-**K**-K-**K**-P-W-W-P-W-R-R-NH_2	1790.9	1791.3
[$L^{8,9}$]Ind	I-L-P-W-K-W-P-**L**-**L**-P-W-R-R-NH_2	1759.9	1761.2
[Y^8,L^9]Ind	I-L-P-W-K-W-P-**Y**-**L**-P-W-R-R-NH_2	1811.4	1811.2
[K^8,L^9]Ind	I-L-P-W-K-W-P-**K**-**L**-P-W-R-R-NH_2	1776.4	1776.2
[$L^{9,11}$]Ind	I-L-P-W-K-W-P-W-**L**-P-**L**-R-R-NH_2	1761.3	1761.2
[L^9,Y^{11}]Ind	I-L-P-W-K-W-P-W-**L**-P-**Y**-R-R-NH_2	1811.5	1811.2
[L^9,K^{11}]Ind	I-L-P-W-K-W-P-W-**L**-P-**K**-R-R-NH_2	1776.4	1776.2
[$K^{4,8}$,L^9]Ind	I-L-P-**K**-K-W-P-**K**-**L**-P-W-R-R-NH_2	1717.6	1718.2
[$K^{6,8}$,L^9]Ind	I-L-P-W-K-**K**-P-**K**-**L**-P-W-R-R-NH_2	1717.1	1718.2
[L^9,$K^{12,13}$]Ind	I-L-P-W-K-W-P-W-**L**-P-W-**K**-**K**-NH_2	1778.8	1778.1
retro-Ind	R-R-W-P-W-W-P-W-K-W-P-L-I-NH_2	1907.2	1907.3
retro-[$L^{8,9}$]Ind[b]	R-R-W-P-**L**-**L**-P-W-K-W-P-L-I-NH_2	1761.7	1761.2
retro-[$K^{4,8}$,L^9]Ind[b]	R-R-W-P-**L**-**K**-P-W-K-**K**-P-L-I-NH_2	1715.6	1718.2
retro-[$K^{6,8}$,L^9]Ind[b]	R-R-W-P-**L**-**K**-P-**K**-K-W-P-L-I-NH_2	1719.6	1718.2

[a] Bold letters present the amino acid residues in analogs different from those in Ind.
[b] The nomenclature of the retro Ind analogs was defined; for example, the peptide denoted as *retro*-[$L^{8,9}$] Ind designates the retro Ind analog, in which Leu is placed at positions 8 and 9 of the parent Ind.

as possible for the purpose of easy handling during preparation and conservation. For systematic screening of the antibacterial and hemolytic activities of peptides, we synthesized a series of analogs, in which one or some Trp residues were substituted with Leu, Lys, Phe and/or Tyr (Table 1). A preliminary study demonstrated an interesting result that [L^9]Ind and [L^{11}]Ind exhibited high antibacterial activity and very low hemolytic activity [29].

Therefore, we designed several more analogs, in which two or three of five Trp residues were replaced by Leu, Tyr, or Lys. [L^9, $K^{12,13}$]Ind was also prepared to investigate the effect of Arg substitution with Lys on biological activities. In addition, we prepared the retro analogs of Ind to investigate their antibiotic and hemolytic activities. The retro analog of Ind, *retro*-Ind, has already been synthesized and the correlation between its secondary structure and the antibacterial activity has been investigated by comparing with those of Ind, however, the hemolytic activity of *retro*-Ind has not yet been investigated [30].

In this study, the secondary structures of the analogs were analyzed on the basis of their CD spectra and their biological activities were evaluated by antibacterial and hemolytic assays. The correlations between the amino acid sequence of the analogs and their antibacterial and hemolytic activities are discussed.

Materials and Methods

Fmoc-amino acids and coupling reagents were purchased from Watanabe Chemical Ind. Ltd. and Calbiochem-Novabiochem AG. Fmoc-aminomethyl-3,5-dimethoxyphenoxy-valeric acid (PAL) resin, 4-(2′,4′-dimethoxyphenylaminomethyl) phenoxy resin (Rink amide resin), was purchased from Applied Biosystems Japan, Inc. The solvents and reagents used for synthesis were supplied by Nakalai Tesque Inc (Kyoto, Japan)., and Wako Pure Chemical Ind. Ltd (Osaka, Japan). Human blood samples were taken from us.

Peptide Synthesis

Ind and its analogs were synthesized from Fmoc-amino acids and Fmoc-PAL-PEG-polystyrene (PS) resin by the solid-phase method using an automated peptide synthesizer, Pioneer, from Applied Biosystems, Inc., employing the standard Fmoc methodology. The peptides were cleaved from the resin by treatment with trifluoroacetic acid/thioanisole/phenol/water/ethanedithiol in the ratio recommended by Applied Biosystems, Inc. The products were purified by gel chromatography on a Sephadex G-25 open column. The crude peptides was purified reverse-phase HPLC on an Inertosil octa decyl silyl (ODS)-3 column (250 × 4.6 mm, Eluents: A: 0.1%TFA/H_2O, B: 0.1%TFA/CH_3CN, A/B: 100/0% → (30 min) → 0/100%, Flow rate: 1.0 ml min^{-1}, Detector: UV 278 nm) on the HITACHI HPLC system. The purity of the final products was evaluated by analytical reverse-phase-HPLC, and was found to exceed 95%. Matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) mass analysis was performed using a Perseptive BioSystems VOYAGER Delayed Extraction (DE-STR) spectrometer and a matrix, α-cyano-(4-hydroxy)cinnamic acid.

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Circular Dichroism

The CD spectra were recorded on a Jasco J-720 W spectropolarimeter with a thermostated cell holder using a quartz cell with a path length of 1.0 or 0.1 mm. The peptides were dissolved in MeOH, the 5 mM [2-{4-(2-hydroxyethyl)-1-piperazinyl}ethanesulfonic acid (HEPES) –NaOH buffer (pH 7.4), and the buffer containing 3 mM of phospholipid vesicles of dipalmitoylphosphatidylcholine (DPPC). The peptide concentration was 5 μM. Measurements were made at 25 °C. The mean residue ellipticity is given as deg cm^2 $dmol^{-1}$.

Antimicrobial Activity

Method of minimum inhibitory concentration (MIC) is as follows; aliquots of 100 μl of each serially diluted peptide in sterilized distilled water were added to the mixture of 10 μl of bacterial cell suspension (approximately 10^6 colony-forming units/ml) and 90 μL of Mueller–Hinton broth in each well of a flat-bottomed microplate. After incubation overnight at 37 °C, the MIC values were measured at the lowest final concentration where no growth was observed. We did antimicrobial activity tests for 7–10 times and obtained within the limits of error the same values for each peptide.

Hemolytic Activity

NaCl (150 mM)/HEPES –NaOH (5 mM) (pH 7.4, 2 ml) was added to fresh human blood (2 ml). The resulting mixture was centrifuged at 3000 rpm for 5 min at 4 °C, and the precipitates were collected. After being washed with NaCl (150 mM)/HEPES –NaOH (5 mM) (pH 7.4) three times, the obtained precipitates (2 ml) were suspended in NaCl (150 mM)/HEPES –NaOH (5 mM) (18 ml) and stored at 4 °C. NaCl (50mM)/HEPES –NaOH (5mM) (pH 7.4, 1800 μl) was added to the human erythrocyte (10% hematocrit) solution (200 μl), followed by peptide in HEPES –NaOH buffer of several concentrations. The resulting suspension was incubated for 30 min at 37 °C, and then centrifuged at 3000 rpm for 5 min at 4 °C. The supernatant was monitored at 540 nm on a Hitachi U-2000 spectrophotometer (A_1). To measure the absorbance for 100% activity (A_2), 10% Triton X-100 (10 μl), instead of a peptide, was added to the erythrocyte in HEPES –NaOH buffer.

$$\text{Hemolysis (\%)} = A_1/A_2 \times 100.$$

The hemolytic activities of retro Ind and analogs, *retro*-[$L^{8,9}$], *retro*-[$K^{4,8}$, L^9], and *retro*-[$K^{6,8}$, L^9]Ind, – the peptide denoted as *retro*-[$L^{8,9}$] Ind designates the retro Ind analog, in which Leu is placed at positions 8 and 9 of the parent Ind – were assayed by a similar method, but a microplate reader was used for only these analogs to save the amount of the samples [31]. The hemolytic assay was carried out for two or three times using the same human blood in each analog for the purpose of confirming the reproducibility. In order to compare these hemolytic activities of Ind and analogs, complete lysis after 0.5 h was normalized in a way that that of Ind (50 μM) is 100.

Results

Preparation of Ind Analogs

We designed a number of peptides by paying attention to the following structural and/or electrostatic features of the residues. The peptides are divided into three groups as follows (Table 1).

In the first group, we tried to know which Trp can be exchanged (or which Trp is essential for antibacterial activity), because the Trp residues are light-unstable but are reportedly necessary to interact with the surface of bacterial cell membranes [20]. For this purpose, a series of analogs in which Trp was substituted with Leu at position 4, 6, 8, 9, or 11 was synthesized. In addition, we tried to verify which role of the Trp residue (i.e. large aromaticity, enabling π-cation interactions with Arg residues; or hydrophobicity, stabilizing the amphiphilic structure [32]) is necessary for antimicrobial activity. Thus, all of the five Trp residues were substituted with similar aromatic Phe or Tyr, or hydrophobic Leu.

In the second group, peptides exhibiting high antibacterial and low hemolytic activity in the first screening were modified by substituting them with one or two additional Trp or Arg residues for the purpose of screening those exhibiting higher bacterial cell lysis and lower hemolysis. As shown below, the Leu-substituted peptide at position 9 was found to exhibit desirable activities in the first screening, and Arg or other Trp residues were substituted with Leu, Tyr, or Lys in this group. Substitution of Trp with Lys was planned to evaluate the effect of more cationic charges in the peptide on the antibacterial activity than that in the original one.

In the third group, Ind analogs having the amino acid sequence in retro order were synthesized, and their biological activities were examined. A retro peptide is generally considered to have similar chemical and structural properties. Nagpal *et al*. already showed that *retro*-Ind and Ind had similar structures/antibacterial activities [30]. However, the terminal structural exchange of amino- and amide-moieties between Arg and Ile might have some influence on the interaction with the cell membrane surface. We, therefore, expected that the *retro*-Ind analogs may perform distinct antimicrobial and/or hemolytic activities from those of the original peptide. All these peptides were synthesized by the common solid-state synthetic method, and identified by MALDI-TOF MS.

Circular Dichroism

CD for Ind and [L^9] Ind, was measured in MeOH, HEPES buffer, and that in the presence of DPPC (Figure 1(A) and (B)). These spectra showed that the secondary structures of both the peptides, random-coil patterns like that of Ind reported previously [33–35], do not depend on the solvents and other molecules around the peptides. The CD spectra for the other analogs in MeOH were also shown in Figure 2(A)–(F). The spectra for Ind and the mono-substituted analog, [L^6]Ind, showed typical random-coil patterns [24], whereas the spectrum of [L^4]Ind exhibited a pattern containing a small β-sheet structure with a minimum around 220 nm (Figure 2(A)). Characteristic positive signals around 230 nm were observed in the spectra for [L^9]Ind and the other [L^9]Ind analogs (Figure 2(A)–(F)), indicating that these molecules especially form some unordered conformation [24]. The spectra for the retro analogs, *retro*-[$L^{8,9}$]Ind, *retro*-[$K^{4,8}$,L^9]Ind and *retro*-[$K^{6,8}$,L^9]Ind, gave similar patterns to those for the corresponding original peptides (Figure 2(E) and (F)), indicating that the CD spectra expose no clear structural difference in these retro peptides from the original ones. The spectrum for [$L^{4,6,8,9,11}$]Ind also showed a different pattern from that of the random coil (Figure 2(B)). In contrast, [$F^{4,6,8,9,11}$]Ind had the α-helix structure, whereas [$Y^{4,6,8,9,11}$]Ind showed the typical β-sheet structure. As shown in Figure 2(C)–(E), the spectra for most of the di- and

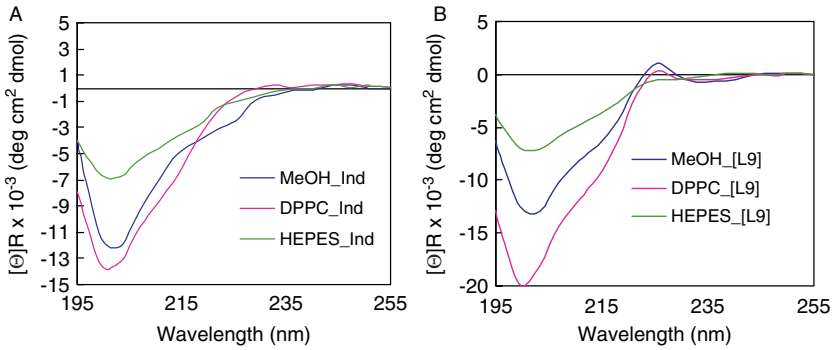

Figure 1. CD spectra for (A) Ind and (B) [L[9]]Ind in MeOH, HEPES buffer, and that in the presence of DPPC.

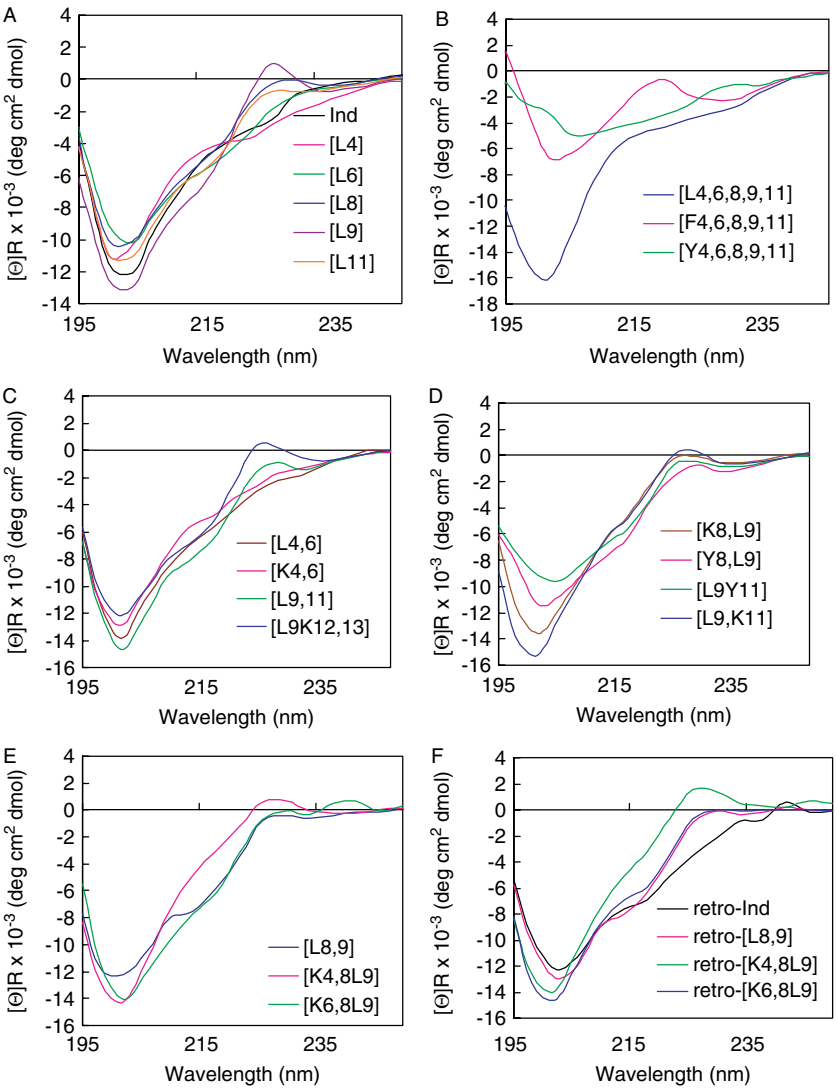

Figure 2. CD spectra for Ind and its analogs in MeOH at 25 °C (A) 1-Residue-substituted Ind, (B) 5-Residue-substituted Ind, (C) 2- or 3-Residue-substituted Ind, (D) 1-Residue-substituted Ind with Leu at position 9, (E) 2- or 3- Residue-substituted Ind, (F) 2- or 3-Residue-substituted retro Ind and its analogs.

tri-substituted analogs, [L[4,6]]Ind, [L[9,11]]Ind, [K[8],L[9]]Ind, [L[9],K[11]]Ind, [K[4,8],L[9]]Ind and [L[9],K[12,13]]Ind showed the typical random-coil patterns, whereas those for the other peptides, [K[4,6]]Ind, [L[8,9]]Ind and [K[6,8],L[9]]Ind, indicated structures containing the small β-sheet, different from those of the other random-coil peptides to some extent.

Antibacterial Activity

The MIC values of Ind and the analogs are shown in Table 2. All the peptides listed in Table 1 exhibited antibacterial activities against Gram-positive bacteria as well as Ind. Interestingly, [L[9]]Ind demonstrated stronger activity than Ind against both

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Table 2. Antibacterial activity of Ind and its analogs

Peptides	MIC (μg ml^{-1})[a] *Bacillus subtilis* IFO 3513	*Staphylococcus aureus* IFO 12732	*Escherichia coli* IFO 12734	Hemolysis[b]
Ind	3.13	1.56	25.0	100
[L^{4}]Ind	3.13	3.13	25.0	73
[L^{6}]Ind	3.13	1.56	25.0	88
[L^{8}]Ind	3.13	1.56	12.5	63
[L^{9}]Ind	1.56	1.56	12.5	6
[L^{11}]Ind	3.13	3.13	25.0	13
[L^{4,6,8,9,11}]Ind	6.25	3.13	25.0	100
[F^{4,6,8,9,11}]Ind	12.5	6.25	100.0	6
[Y^{4,6,8,9,11}]Ind	25.0	25.0	>100.0	4
[L^{4,6}]Ind	3.13	3.13	50.0	8
[K^{4,6}]Ind	6.25	6.25	100.0	8
[L^{8,9}]Ind	6.25	6.25	25.0	18
[Y^{8}, L^{9}]Ind	3.13	6.25	25.0	60
[K^{8}, L^{9}]Ind	3.13	3.13	25.0	0
[L^{9,11}]Ind	50.0	25.0	>100.0	3
[L^{9}, Y^{11}]Ind	3.13	6.25	50.0	12
[L^{9}, K^{11}]Ind	3.13	6.25	50.0	3
[K^{4,8}, L^{9}]Ind	3.13	6.25	50.0	7
[K^{6,8}, L^{9}]Ind	1.56	3.13	12.5	11
[L^{9}, K^{12,13}]Ind	3.13	3.13	50.0	8
retro-Ind	1.56	1.56	12.5	36
retro-[L^{4,8}]Ind	6.25	6.25	25.0	11
retro-[K^{4,8}, L^{9}]Ind	6.25	6.25	50.0	9
retro-[K^{6,8}, L^{9}]Ind	12.5	12.5	50.0	7

[a] Reported values are averages of 7–10 independent determinations for each peptide. Antimicrobial-activity tests were carried out for 7–10 times and the same results were obtained in each peptide.
[b] Complete lysis after 0.5 h was normalized in a way that that of Ind (50 μM) is 100.

Gram-positive and Gram-negative bacteria. Other Leu-containing analogs in Table 1 also exhibited antibacterial activities similar to Ind, suggesting that any Trp residue in Ind can be replaced with bulky aliphatic amino acids. When all of the five Trp residues were substituted with Leu, the activity toward Gram-negative bacteria did not decrease. Therefore, we speculated that hydrophobicity in the whole peptide is essential for acting as a cell-lysing peptide at least in this case. Substitution of all the Trp residues with Phe or Tyr residues decreased antibacterial activity against Gram-positive bacteria and resulted in the loss of activity against Gram-negative bacteria.

The analog containing two 12,13-Lys residues, [L^{9},K^{12,13}]Ind, also had antimicrobial activity toward both Gram-positive and Gram-negative bacteria, supporting the view that the positive charge at the terminal position derived from Arg or Lys residue is required for antibacterial activity [23]. Among the other di- or tri-substituted analogs, the series of 6-, 8- and 9-Trp-substituted peptides of Leu, Lys, or Tyr, such as [L^{8,9}]Ind and [Y^{8},L^{9}]Ind, [K^{8},L^{9}]Ind, and [K^{6,8},L^{9}]Ind, performed high activities against Gram-positive and Gram-negative bacteria. Remarkably, the activity of [K^{8},L^{9}]Ind was similar to that of Ind. Further, [K^{6,8},L^{9}]Ind exhibited higher antibacterial activity than Ind. On the other hand, the di- or tri-substituted analogs containing Leu, Lys, or Tyr at position 4 or 11, such as [K^{4,6}]Ind, [L^{4,6}]Ind, [L^{9,11}]Ind, [L^{9},Y^{11}]Ind, [L^{9},K^{11}]Ind and [K^{4,8},L^{9}]Ind, showed low activity especially against Gram-negative bacteria, except for mono-substituted [L^{4}]Ind and [L^{11}]Ind having high activities. Comparison of the substituted positions of highly active peptides with those of less active peptides indicated that only the two Trp residues at positions 4 and 11 in Ind play important roles in the lysis action against at least Gram-negative bacteria, and the other Trp residues can be substituted for designing antibacterial peptides. In addition, in the cases of mono-substituted [L^{4}]Ind and [L^{11}]Ind, the nearby Trp residues might compensate for the loss of the interactions with the cationic residues, accompanied by some conformation change.

The retro peptide of Ind had high antibacterial activity (Table 2), as reported in the literature [30]. However, the retro analog of [K^{6,8},L^{9}]Ind showed considerably weaker activities than those of the original analog.

Hemolytic Activity

The lysis of erythrocytes as one of the functions of Ind and substituted analogs was investigated. The hemolytic action of these peptides is regarded as a negative function of antimicrobiol peptides, fatally risky when they are used for pharmaceutical applications. Interestingly, in contrast to the hemolytic activity of Ind, that of some analogs was reduced by substitution of a Leu residue for one of the five Trp residues. As shown in Table 2, the hemolytic activities of the analogs were standardized, as the hemolysis of Ind (50 μM) after 30 min is 100. In particular, the activity of [L^{9}]Ind and [L^{11}]Ind clearly decreased. Other Leu-containing analogs had slightly or fairly weaker activities compared to Ind. This result suggests that both the Trp residues at positions 9 and 11 strongly interact with the cationic residues to perform the hemolytic activity. [L^{4,6,8,9,11}]Ind showed the similar hemolytic activity to that of Ind, but [F^{4,6,8,9,11}]Ind and [Y^{4,6,8,9,11}]Ind exhibited low activity.

The hemolytic activities of the di- or tri-substituted Trp and/or Arg analogs are shown in Table 2. [K^{8},L^{9}]Ind and [L^{9},K^{11}]Ind exhibited negligible hemolytic activities, whereas the corresponding analogs substituted by Tyr, [Y^{8},L^{9}]Ind and [Y^{9},K^{11}]Ind showed higher activities. Low hemolytic activities were also performed by [K^{6,8},L^{9}]Ind, which has high antibacterial activity, and the other peptides, in which three Trp residues were substituted (Table 2). More noticeable is that the hemolytic activity of the retro analog of Ind is about 1/3 of the original one. Such a clear difference in the biological activity between the retro and original peptide has never been reported. Although the reason for the phenomenon is unknown, this might be attributable to the terminal substituent exchange, especially at the Arg residue, which plays an important a role in erythrocyte-membrane interaction. The other retro analogs, *retro*-[L^{8,9}]Ind, *retro*-[K^{4,8},L^{9}]Ind and *retro*-[K^{6,8},L^{9}]Ind also have low hemolytic activities. This information is significant and may indicate that the use of retro analogs of small peptides, such as antibacterial Ind containing Trp and Arg residues, could be an efficient approach to suppress the hemolytic activity.

Discussion

The relationships of the structural features of Ind and functional properties of Trp with cell-lysis activities can be derived from the evaluations of a series of Ind analogs: their CD spectral patterns, antibacterial activities against Gram-positive and Gram-negative

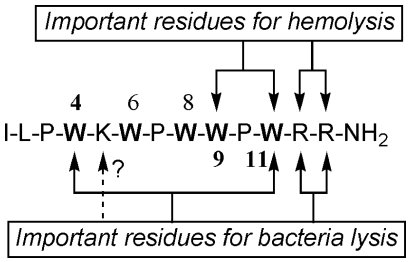

Figure 3. Primary structure-activity relationship of Ind.

bacteria, and hemolytic activities. The secondary structures of the Ind analogs are not significantly different from the random-coil structures of the original peptide. We found that β-sheet structures in the analogs, attributed to the presence of Tyr residues, did not clearly affect the antibacterial and hemolytic activities, as P. R. Hansen *et al*. described in the literature [25].

According to the MIC results of Ind and the analogs, antibacterial activity against Gram-negative bacteria was low in the Trp analogs substituted at positions 4 and/or 11 except for [$L^{4,6,8,9,11}$]Ind. On the other hand, a Trp analog substituted at positions 6, 8, and 9, which are the others of the five Trp residues, exhibited the highest activity, showing the very important roles of Trp residues at positions 4 and 11 for the antimicrobial activity of Ind (Figure 3). The analog, of which all Trp residues were substituted with Leu, [$L^{4,6,8,9,11}$]Ind, exhibited high activities toward both Gram-negative bacteria and erythrocyte, suggesting that the analog plays the cell-lysis action in other mechanism (utilizing hydrophobicity, stabilizing the amphiphilic structure) [36] than the antimicrobial Trp peptides.

The important residues of Ind in hemolysis are not the same as those in bacterial cell lysis, interestingly. The hemolytic activity of the Trp analogs at position 9 or 11 decreased dramatically, showing that the pair of Trp residues is essential for lysis of human blood cells (Figure 3). This result indicates that substitution at position 9 or 11 in the Trp analog may be effective in suppressing hemolysis. However, the Trp residue at position 11 is also essential for the interaction of Ind with bacterial cell membranes, i.e. substitution of the Trp residue at position 9 in Ind is the key to deactivating hemolysis while maintaining its antibacterial activity toward Gram-negative bacteria. The Trp residues at positions 6 and 8, which did not participate in biological activities, were found to be free for substitution from the above discussion. The Trp residues at positions 9 and 11 are close to the two terminal Arg residues, being easily accepted as the important positions in the concerted interaction of the cell membranes. On the other hand, the significance of the Trp residue at position 4, which is far from the terminal Arg residues in the random-coil structure of Ind, is interesting, suggesting that the Lys residue at position 5 might play some role in bacterial cell lysis (Figure 3) or other mechanism, such as peptide oligomerization, proceeds in hemolysis. This proposal now has no direct evidence and needs further investigation.

Conclusion

In summary, a series of Trp and Arg analogs of antibacterial Ind was synthesized and the antimicrobial and hemolytic activities thereof were investigated. [L^{9}]Ind, [L^{11}]Ind, [K^{8},L^{9}]Ind and [$K^{6,8}$,L^{9}]Ind showed desirable characteristics, exhibiting negligible hemolytic activity while keeping strong antibacterial activity. We found that the Trp residue at position 11 essentially contributes to both activities and it cannot be exchanged for the other, whereas the Trp residues at positions 4 and 9 play important roles in antimicrobial and hemolytic activities, respectively. The Trp residues at positions 6 and 8 play no important roles in biological activities. We then found that the retro analog of Ind showed higher antibacterial activity than Ind against both Gram-positive and Gram-negative bacteria but remarkably lower hemolytic activity (1/3) than that of Ind. These results will benefit the chemistry of antibiological peptides and their applications, and we are now investigating the detailed phenomena in the perturbation processes on the cell membranes of bacteria and blood with these Ind analogs.

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