Journal of Peptide Science
J. Peptide Sci. **7**: 552–564 (2001)
DOI: 10.1002/psc.351

Structure–Function Relationships in the Tryptophan-rich, Antimicrobial Peptide Indolicidin

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Received 15 May 2001
Accepted 20 June 2001

Abstract: Indolicidin is a cationic 13 amino acid peptide amide produced in the granules of bovine neutrophils with the sequence H-ILPWKWPWWPWRR-NH_2. Indolicidin is both antimicrobial and, to a lesser extent, haemolytic. In order to systematically investigate structure–function relationships, the solid-phase synthesis of indolicidin and 48 distinct analogues are reported, as well as the characterization of their respective biological properties. Peptides synthesized and characterized include analogues with modified terminal functions, truncations from either terminus, an alanine scan to determine the role of each individual amino acid, specific amino acid exchanges of aromatic, charged and structural residues and several retro-, inverso- and retroinverso-analogues. Together, characterization of these analogues identifies specific residues involved in antimicrobial or haemolytic activity and suggests a core structure that may form a scaffold for the further development of peptidomimetic analogues of indolicidin. Copyright © 2001 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: antimicrobial peptide; defence peptide; indolicidin; indolicidin analogues; inverso-peptide; retro-peptide; structure–function relationships

INTRODUCTION

Indolicidin is a 13 amino acid antimicrobial peptide amide, originally isolated from the granules of bovine neutrophils [1]. The sequence of indolicidin (H-Ile-Leu-Pro-Trp-Lys-Trp-Pro-Trp-Trp-Pro-Trp-Arg-Arg-NH_2) is of considerable interest since it contains the highest known percentage content of Trp of any natural protein or peptide. Moreover, the structural gene encoding indolicidin production in bovine neutrophils has been identified and characterized [2] and the structure of the peptide has been confirmed by synthesis [3]. Indolicidin is just one member of a larger group of antimicrobial peptides (sometimes called defence peptides) produced by eukaryotic cells and which are involved in the maintenance of so-called innate immunity (for reviews see references [4–6]). Such defence peptides can be found in a variety of different epithelial and myeloid cell types and can be isolated from a large range of organisms including invertebrates, vertebrates and plants [7–9]. Moreover, they are also closely related to similar antimicrobial peptides of bacterial origin called bacteriocins [10–12].

Interestingly, indolicidin effectively kills both Gram-positive and Gram-negative bacteria and its antimicrobial activity against a number of bacterial pathogens [13–16], as well as its mode of action

Abbreviations: ES-MS, electrospray ionization mass spectrometry; Pbf, $N\omega$-2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulphonyl.

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against specific bacteria [17–19], has been extensively studied. From these reports, it has been suggested that indolicidin is a membrane active peptide, permeabalizing the cytoplasmic lipid bilayer of sensitive bacteria. However, at least two recent studies have suggested that this mode of action may not completely account for the antibacterial activity of indolicidin [18,19]; although additional mechanisms of killing are not completely clear. Many other antimicrobial peptides isolated from a variety of sources have been shown to have a membrane-permabilizing mode of action. In some cases, such as those of the lantibiotics nisin and epidermin [20,21] and the bacteriocin pediocin PA-1 [22], this mechanism has been shown to be dependent on the presence of a 'docking molecule' or receptor-like molecule present in the susceptible bacteria and which is necessary for full antimicrobial activity; it is not currently known if indolicidin activity is facilitated by a similar mechanism. In addition to its antimicrobial activity, indolicidin is also haemolytic and has been suggested to be antiviral, at least against HIV-1 and herpes simplex [23,24].

Not all bacteria are equally sensitive to defence peptides. Recently, we have used molecular genetic modification to identify two bacterial gene products that contribute to bacterial resistance to defence peptides, both of which are involved in modifications to the cell envelope of the Gram-positive pathogen *Staphylococcus aureus* and are probably also found in several other bacterial pathogens. Firstly, reduction in the amount of D-Ala esterified to teichoic acids in the cell envelope of *S. aureus* (brought about by disruption of the protein DltA) results in increased negative charge in the envelope and a concomitant increase in electrostatic attraction of cationic defence peptides towards the cell membrane, their site of action [25]. Similarly, the protein MprF, which is an important virulence factor in *S. aureus* (and probably also other pathogenic bacteria), has been shown to be involved in the production of the unusual phospholipid lysylphospatidylglycerol (L-PG) which, because of its basic nature, reduces the overall negative charge of the cytoplasmic membrane, repelling the cationic antimicrobial peptides [26]. Both of these systems demonstrate the importance of electrostatic attractive and repulsive forces in the mode of action of cationic defence peptides and their presence helps to explain the pathogenicity and relative defence peptide insensitivity of *S. aureus*. Moreover, *S. aureus* is an important pathogen of epithelial surfaces (which are normally bathed in defence peptides), post-surgical wounds, indwelling intravascular devices and patients with HIV and AIDS [27]. Thus, characterization of these defence peptide resistance mechanisms has identified targets for antimicrobial therapy that could render the bacteria sensitive to the hosts innate immune defence systems and may form the basis for treatment of multidrug-resistant *S. aureus* infections. Alternatively, development of defence peptide analogues that are effective even in the presence of pathogenic factors such as MprF may provide the basis for the development of a new generation of antimicrobial therapeutics.

Several analogues of indolicidin have already been reported in an attempt to better understand the mechanism of action of the peptide and to elucidate the nature of its secondary structure. For example, it has been shown that a single Trp-Trp cross-link may stabilize the structure of indolicidin against proteases, without significantly affecting the biological activity [28]. Replacement of various residues has been shown to greatly affect the biological activity of indolicidin [6,29–31]; although replacement of Trp with Phe appears to yield an analogue with similar antimicrobial activity to natural indolicidin, whilst significantly reducing the haemolytic activity of the peptide. Moreover, controversy over the secondary structure of indolicidin has also prompted the synthesis of several analogues [17,32]; initially, indolicidin was thought to consist mainly of poly-proline helix, but subsequent study has suggested its secondary structure is the result of a series of Type VI turns generated by stacking of *cis*-proline residues with neighbouring aromatic amino acid side-chains. Other studies have addressed this same issue by investigating the structure of indolicidin in the presence of micelles of various compositions [6,33]. By NMR analysis of the bilayer-localized structure, it has been suggested that indolicidin forms an unique structure, different from the solution structure of the peptide (i.e. in the absence of phospholipid micelles) and which consists of two half-turns, with the indole side-chains folded flat against the peptide backbone to produce an overall wedge shape [34].

Here we present the first systematic survey of the biological activities of a range of synthetic indolicidin analogues, including those with altered terminal functions, altered chiral structure, sequence inversion, individual alanine replacements and specific substitutions of aromatic, charged and potentially structural amino acids. The results obtained give us new insight into the

structure-function relationships of indolicidin and define residues and structure or physicochemical features of the peptide necessary for antimicrobial activity against Gram-positive or Gram-negative bacteria, as well as haemolytic activity against human erythrocytes. Moreover, they suggest a possible scaffold structure for the development of peptidomimetics of indolicidin with favourable biological properties that might be further investigated for topical treatment of *S. aureus* infection.

MATERIALS AND METHODS

Peptide Synthesis

Peptides were synthesized using the Fmoc-protection strategy and appropriate methods for multiple parallel solid-phase peptide synthesis on 1% divinylbenzene cross-linked polystyrol modified with a Rink-linker for the synthesis of peptide amides (0.52 mmol/g; PepChem, Tübingen, Germany) or Fmoc-Arg(Pbf)-preloaded tritylchloride-polystyrol (TCP) resin for synthesis of peptide acids (0.48 mmol/g; PepChem). $N\alpha$-Fmoc protected amino acids were purchased from NovaBiochem (Bad Soden, Germany) and specific side chain functions were further protected as follows: Asp, Glu, Ser and Tyr, *t*Bu; His, Trt; Lys, lys, Orn, Trp and trp, Boc, Arg and arg, $N\omega$-2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulphonyl (Pbf). Protected amino acids were coupled twice in 5-fold excess (with respect to initial resin loading) using diisopropylcarbodiimide (DIC) in the presence of 1-hydroxybenzotriazole (HOBt) over 90 min and a capping step utilizing 10% (v/v) acetic anhydride/5% (v/v) DIEA in DMF for 15 min was included after each coupling to minimize the complexity of failure sequences. Complete Fmoc deprotection was facilitated by the use of 25% (v/v) piperidine in DMF, once for 3 min and subsequently with two 12-min applications. Peptide resin was washed after each step in both DMF and dichloromethane:DMF (3:2) as appropriate. The $N\alpha$-acetylated analogue (Ac-indolicidin) was prepared in the same way except that its synthesis included an additional capping step after removal of the $N\alpha$-Fmoc protection prior to cleavage and deprotection. Peptides were cleaved from the resin and deprotected in a mixture of (v/v) 92.5% trifluoroacetic acid/5% triisopropylsilane/2.5% water within 3 h and were then precipitated from ice-cold ether, recovered by centrifugation and decantation of the ether phase and lyophilized twice from 80% (v/v) aq. 2-methyl-propan-2-ol to yield the final crude product. All reagents and solvents (except where otherwise stated) were purchased from Sigma-Aldrich-Fluka Chemie, Steinheim, Germany.

Purification and Characterization of Peptides by RP-HPLC

The purity of analogues was assessed using analytical RP-HPLC in two distinct buffer systems. Briefly, peptides (ca. 1–2 μg) were separated on a 40×2 mm column of 3 μM particle size GromSil ODS-4HE (Grom Analytik and HPLC, Herrenberg, Germany) using appropriate binary solvent gradients (normally 0–100% B in 5 min) of either (A) water/0.1% TFA and (B) acetonitrile/0.1% TFA or (A) 25mM potassium phosphate buffer (pH 7.0) and (B) 80% aq. acetonitrile with a flow rate gradient of 400 (at 0% B) to 700 (at 100% B) μL/min. Elution was monitored by absorbance at 214 and 280 nm.

Peptides with apparent purities in either buffer system of $< 90\%$ (determined by peak area at 214 nm) were purified by preparative RP-HPLC on a 60×20 mm column of 3 μL particle size GromSil ODS-4HE (Grom Analytik and HPLC) in a binary gradient of 10% aq. acetonitrile/0.1% TFA to 80% aq. acetonitrile/0.1% TFA in 15 min at a flow rate of 30 ml/min. Elution was monitored at 214 nm and peak fractions were collected manually, lyophilized and reanalysed by analytical HPLC as described above.

Analytical Characterization of Indolicidin Analogues

In addition to analysis of the purity of indolicidin analogues (above), the identity of the peptides was determined using ES-MS and, in some cases, chiral phase amino acid analysis and amino acid sequencing. Briefly, mass spectra were collected on a VG Quattro II triple quadrupole mass spectrometer (Micromass) fitted with an atmospheric pressure electrospray ionization source and data were collected and analysed using the manufacturers software (MassLynx v2.2). Samples, dissolved in 50% aq. acetonitrile containing 0.5% (v/v) formic acid at concentrations of ca. 10–50 μg/ml, were introduced at flow rates of 30–60 μL/min in a stream of 50% aq. acetonitrile containing 0.1% (v/v) formic acid and were ionized in air at cone voltages between 30 and 60 kV.

In order to quantitate the degree of amino acid racemization that occurred during synthesis, in particular in the preparation of retro- and retroinverso-analogues, we analysed the amino acid

composition of hydrolysates of indolicidin and a number of its analogues using chiral phase GC-MS [35]. Briefly, samples (ca. 10–20 nmoles) were hydrolysed *in vacuo* overnight at 110°C in 6 M DCl/D_2O containing 20 μM thioglycolic acid, following which the hydrolysates were dried in a stream of N_2. Samples were then successively derivatized by esterification with 20% (v/v) acetylchloride and monodeuteroethanol at 110°C for 30 min and, after removal of the solvent in a N_2 stream, by trifluoroacetylation with trifluoroacetic acid anhydride at 110°C for 10 min. After removal of the solvent (N_2 stream), the samples, dissolved in toluene, were analysed and collected data were processed on a model 5973 gas chromatograph coupled to a model 6890 electron impact (EI) mass spectrometer (Hewlett-Packard) using the manufacturers software and protocols. Separation was achieved by passage through 25 m × 250 μM columns of either fused silica modified with 30% 2,6-dipentyl-3-butyryl-cyclodextrin in PS255 (film thickness of 0.13 μM) or chirasil-Val using appropriate temperature gradients.

Since a number of synthesized analogues, in particular inverso-analogues or those involving alanine substitutions at different positions, share identical masses and similar HPLC retention times, we characterized the synthesized products by peptide sequencing. *N*-terminal amino acid sequence analysis was carried out in a model 477A pulsed gas-liquid phase protein/peptide microsequencer coupled online with a model 420A narrow bore phenylthiohydantoin (PTH) amino acid analyser (Perkin–Elmer/Applied Biosystems, Weiterstadt, Germany). Samples of peptides (ca. 200 pmol) were applied to a glass fibre disk pre-treated with Biobrene plus™ (15 μL) and dried in a nitrogen stream. All chemicals and protocols for peptide microsequencing were supplied by the instrument manufacturer and data were collected and analysed using the manufacturers software.

Determination of the Minimum Inhibitory Concentration (MIC)

MIC values were determined in triplicate in a standardized microtitre plate assay. Briefly, doubling dilutions of peptide stock solutions dissolved in PBS were prepared in Antibiotic Test Medium 3 (Difco) in a final volume of 100 μL in flat-bottom microtitre plates (Greiner Labortechnik, Frickenhausen, Germany) and 100 μL of the same medium inoculated with sufficient bacterial cells to give a final OD_{578} of 0.04 was then added. The plates were incubated on a rotary shaker (220 rpm) in an humidified chamber at 35°C for 18 h, after which the optical density of the bacterial cultures was determined in an ELISA-plate reader (SpectraMax TM340, Molecular Devices, Sunnyvale, CA, USA) at 578 nm. The MIC was defined as the concentration of the peptide inhibiting the growth of the bacteria by 90% as compared to the control to which no peptide was added. The MIC value of each analogue was determined against *S. aureus* Newman (wild-type pathogen), *S. aureus* Newman ΔMprF (a mutant of the wild-type strain lacking *mprF* and which is therefore hypersensitive to the action of a variety of cationic antimicrobial peptides) [26] and *Escherichia coli* DH5α [36]. All strains were routinely cultured in Antibiotic Test Medium 3 and were propagated and maintained on the same medium supplemented with 1.2% (w/v) agar (Difco).

Determination of the Minimum Haemolytic Concentration (MHC) and Haemolytic Index (HI)

The minimum haemolytic concentration was determined by preparing (in triplicate) doubling dilutions of peptides in PBS (starting from a highest concentration of 1000 μM) in 100 μL volumes in round-bottom microtitire plates to which 50 μL of washed (PBS) human erythrocytes (diluted 1:50 in PBS) were added. The erythrocytes were incubated overnight at 4°C in an humidified chamber and the MHC, defined as the minimum concentration required to cause complete haemolysis of the erythrocytes, was determined visually. Concomitantly, 20 μL aliquots of doubling dilutions of the peptide analogues (starting from a highest concentration of 100 μM), prepared in PBS, were spotted onto Luria-Bertani (LB) agar plates seeded with the sensitive indicator strain *M. luteus* ATCC 9341, allowed to dry in to the agar and the plates were incubated overnight at 35°C. The haemolytic index was defined as the MHC of a given analogue divided by the minimum concentration required for complete inhibition of the growth of *M. luteus* in the plate diffusion assay.

RESULTS

Peptide Synthesis and Characterization of Peptides

Using the procedure given in the materials and methods, we synthesized both indolicidin and the 48 analogues reported here. In each case the

desired peptides could be identified by ES-MS and characterization by RP-HPLC in two different buffer systems showed that most had purities in excess of 90% directly after synthesis and work-up. Notable exceptions were the analogues [Trp4,6,8,9,11His]-indolicidin (68%), [Lys5Ala]-indolicidin (73%), [Trp4,6,8,9,11Asp]-indolicidin (54%) and [Trp4,6,8-9,11Glu]-indolicidin (59%). These analogues, together with several others whose purities lay between 85 and 90%, were purified prior to testing. Specific synthesis failure products were not further analysed. Chiral phase amino acid analysis of various analogues showed that those synthesized from D-amino acids contained >98% D-stereoisomer whilst indolicidin contained >98% L-amino acid and confirmed that the amino acids were present in the expected relative amounts. Moreover, Edman sequencing of several analogues, including indolicidin and [Trp4,6,8,9,11Phe]-indolicidin and each of their retro-, inverso- and retroinverso-analogues, confirmed the expected sequences had been synthesized.

Antimicrobial Activity of Synthetic Indolicidin Analogues

A number of indolicidin analogues of varying length and with different amino or carboxy-terminal functions were synthesized and tested for biological activity (Figure 1). Both Ac-indolicidin and indolicidin-OH showed considerably reduced antimicrobial activity, as did a number of other analogues of decreasing length. Interestingly, indolicidin (2–13) was about 2-fold more active than the parent peptide. Similarly, indolicidin (4–13) and indolicidin (5–13) were approximately as active as the parent peptide, although the analogue indolicidin (3–13) showed considerably diminished activity against all three bacterial strains. Deletion of the *C*-terminal, positively charged amino acids essentially abolished the antimicrobial activity against *S. aureus* wild-type and *E. coli*, but further deletions had a much lesser effect when tested against these strains; surprisingly, an analogue consisting of only the first eight amino acids retained antimicrobial activity against the tested strains.

In order to assess the role of each individual amino acid within the indolicidin structure, we performed an alanine scan in which each residue was individually replaced by the small, neutral amino acid alanine (Figure 2). Exchange of residues Leu2 and Pro3 had no significant effect on the antibacterial activity, whilst the exchanges [Trp8Ala]- and [Trp9Ala]-indolicidin only affected a reduction of the activity against the *S aureus* wild type. However, replacement of the *N*-termimal Ile, the deletion of which (above) results in improved biological activity, resulted in an approximately 2-fold increase in MIC and the exchange Lys5Ala raised the MIC against

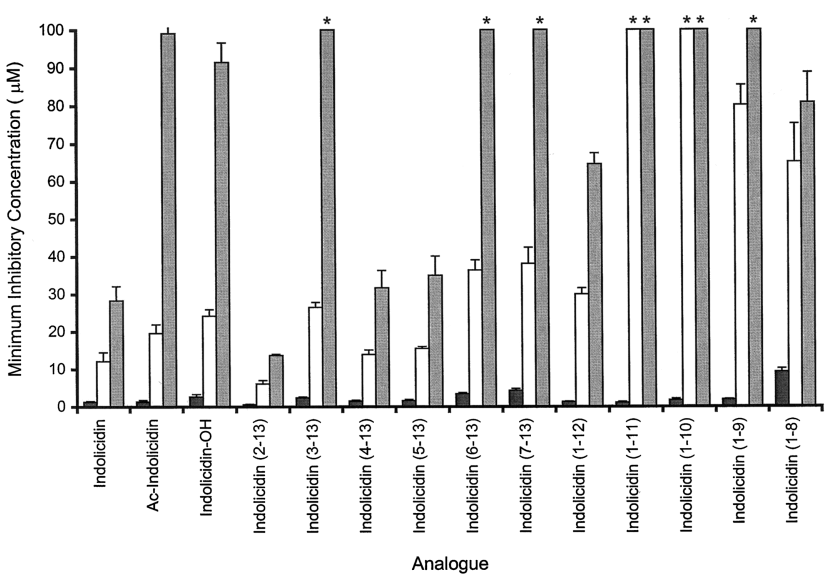

Figure 1 MIC of various indolicidin analogues incorporating modified *N*- or *C*-terminal functions or truncations from either terminus tested against (filled) *S. aureus* Newman ΔMprF, (unfilled) *S. aureus* Newman and (grey-shaded) *E. coli* DH5α. Analogues for which no MIC could be determined (i.e. MIC > 100 μM) are indicated by an asterisk. Results are the mean of triplicate measurements and standard deviation is indicated where appropriate.

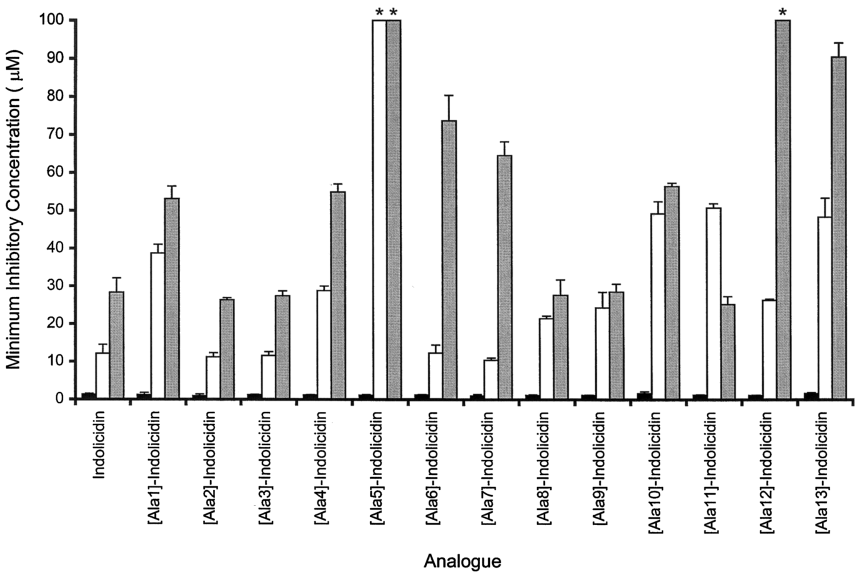

Figure 2 MIC of indolicidin analogues in which each residue was sequentially and individually exchanged with alanine (alanine-scan) tested against (filled) *S. aureus* Newman ΔMprF, (unfilled) *S. aureus* Newman and (grey-shaded) *E. coli* DH5α. Analogues for which no MIC could be determined (i.e. MIC > 100 μM) are indicated by an asterisk. Results are the mean of triplicate measurements and standard deviation is indicated where appropriate.

both *S. aureus* wild type and *E. coli* above the detection limit, although antimicrobial activity against the MprF-deficient mutant remained unaffected. Other exchanges also resulted in altered specificity for either the Gram-negative or Gram-positive bacteria. The exchanges Trp6Ala and Pro7Ala had minimal effect on the antimicrobial activity against *S. aureus* wild-type but affected a 2-fold reduction in activity against *E. coli*. Conversely, Trp11Ala was 2–3-fold less active against *S. aureus* wild type, while retaining essentially unaltered activity against *E. coli*. None of the alanine replacement analogues displayed significantly altered antimicrobial activity against the MprF-deficient *S. aureus* mutant.

Replacement of specific amino acids with a variety of alternatives also revealed interesting features of indolicidin activity (Figure 3). Complete replacement of Trp residues with Phe ([Trp4,6,8,9,11Phe]-indolicidin), completely replacing Pro residues with Gly, exchange of the positively charged Lys5 with either Arg or Orn, or replacing the *C*-terminal pair of Arg residues with Lys had little or no effect on antibacterial activity. Conversely, exchange of the *C*-terminal Arg residues with Cit, replacing the positively charged Lys and Arg residues with either Asp or Glu or the total replacement of Trp residues with either Val, Ser, His, Lys or Glu resulted in abolition of observable antibacterial activity; Trp replacement with either Tyr, Leu or Ile also resulted in significant reduction in the antimicrobial activity against all of the strains tested.

In order to assess the likely role of a receptor-mediated mechanism of action for indolicidin, we synthesized the inverso-, retro- and retroinverso-analogues of both indolicidin and [Trp4,6,8,9, 11Phe]-indolicidin (Figure 4). In each case, the retro-, inverso- and retroinverso-analogues all retained approximately the same antibacterial activity as their respective parent peptides, suggesting that both chiral and sequence-specific recognition of the peptides is unlikely to play a role in their modes of action.

Haemolytic Activity of Synthetic Indolicidin Analogues

Since indolicidin is known to be haemolytic, we also assessed the haemolytic activity of each analogue using human erythrocytes (Table 1). Since some peptides show a concomitant reduction in both antimicrobial activity and haemolysis while others remain unaffected in one or other of the biological activities, we calculated the HI. This value compares the haemolytic activity (MHC) of a given analogue with the MIC determined against the sensitive indicator *M. luteus* in a plate diffusion assay and is more useful for comparison of analogues since it is

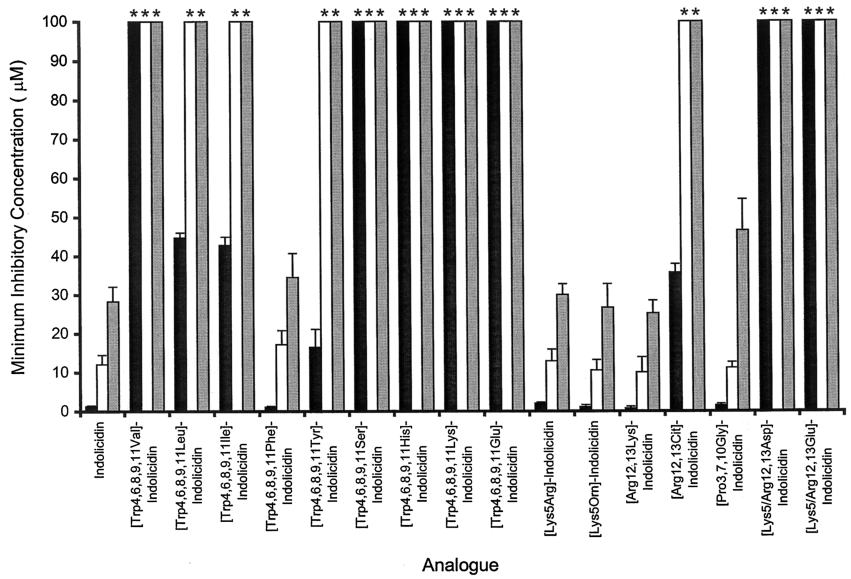

Figure 3 MIC of various indolicidin analogues in which specific amino acids have been exchanged tested against (filled) *S. aureus* Newman ΔMprF, (unfilled) *S. aureus* Newman and (grey-shaded) *E. coli* DH5α. Analogues for which no MIC could be determined (i.e. MIC > 100 μM) are indicated by an asterisk. Results are the mean of triplicate measurements and standard deviation is indicated where appropriate.

a reflection of the relative 'usefulness' of the analogue (an analogue with high HI is antibacterial but poorly haemolytic).

Modification of the terminal functions revealed that, while *C*-terminal carboxylation and *N*-terminal acetylation have a negative impact on antibacterial activity, their respective haemolytic abilities were not altered (i.e. the HI is reduced). Alternatively, truncations from the *C*-terminus generally lead to both a reduction in antimicrobial activity and a concomitant reduction in haemolysis. The analogue [ΔIle1]-indolicidin is both 2-fold more antimicrobial and 2-fold more haemolytic, however further truncation of the peptide (for example [ΔIle1-Pro3]-indolicidin) results in an analogue which retains similar antimicrobial activity to that displayed by indolicidin, while being approximately 2-fold less haemolytic. Truncation of more than the first four amino acids resulted in a generalized reduction in both antimicrobial and haemolytic activity. Alternatively, none of the alanine replacement analogues displayed altered haemolytic activity, although several analogues have lower HI values because of their reduced activity against *M. luteus*.

Specific amino acid substitutions revealed the degree to which modification of the indolicidin structure differentially affects antimicrobial and haemolytic activity. Several analogues involving global substitution of the aromatic residue Trp, including [Trp4,6,8,9,11Val]-, [Trp4,6,8,9,11Ser]-, [Trp4,6,8,9,11His]-, [Trp4,6,8,9,11Lys]- and [Trp4,6,8,9,11Glu]-indolicidin possessed neither demonstrable antimicrobial, nor haemolytic activity. However, replacement of the aromatic residues with the aliphatic amino acids Leu and Ile reduced the antimicrobial activity approximately 63-fold, while generating only a 4-fold reduction in haemolytic activity. With the exception of the analogue [Trp4,6,8,9,11His]-, which is inactive (above), substitution of Trp residues with other aromatic amino acids ([Trp4,6,8,9,11Tyr]- and [Trp4,6,8,9,11Phe]-) had significant ramifications on the haemolytic activity; the tyrosine-containing analogue displayed both 16-fold reduced antimicrobial activity and 4-fold reduced haemolysis, while the phenylalanine-containing analogue displayed unchanged antimicrobial activity but was about 20-fold less haemolytic; this latter result is in agreement with previously reported data [6,18, 30,31]. Substitutions of basic amino acids with alternative basic amino acids had little affect on their MIC or MHC. However, although the substitution of Arg residues with citrulline affected a marked increase in MIC and MHC, the HI was unaffected, suggesting a generalized disruption of biological activity. Interestingly, the analogue [Pro3,7,10Gly]-indolicidin retains its antibacterial activity, but appears to be approximately 2-fold more haemolytic.

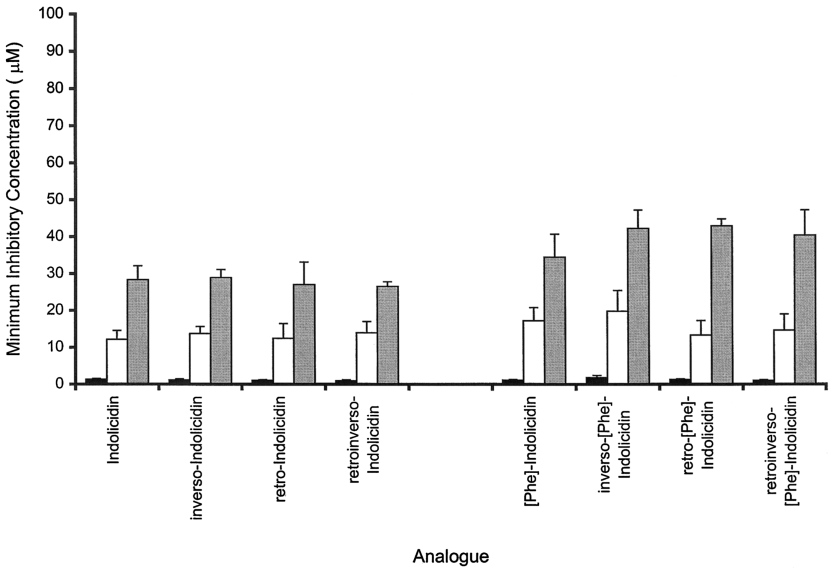

Figure 4 MIC of retro-, inverso- and retroinverso-analogues of indolicidin and [Trp4,6,8,9,11Phe]-indolicidin tested against (filled) *S. aureus* Newman ΔMprF, (unfilled) *S. aureus* Newman and (grey-shaded) *E. coli* DH5α. Analogues for which no MIC could be determined (i.e. MIC > 100 μM) are indicated by an asterisk. Results are the mean of triplicate measurements and standard deviation is indicated where appropriate.

Finally, both the retro-analogues retro-indolicidin and retro-[trp4,6,8,9,11phe]-indolicidin showed unaltered haemolytic activity, suggesting that haemolysis may be non-specific activity, unrelated to the presence of erythrocyte-specific receptors. Interestingly, while sequence inversions (inverso-indolicidin, retroinverso-indolcidin, inverso-[Trp4,6,8, 9,11Phe]-indolicidin and retroinverso-[trp4,6,8, 9,11phe]-indolicidin) all displayed unaltered antimicrobial activity, they may be approximately 2-fold less haemolytic as compared to the respective parent peptides.

DISCUSSION

Antimicrobial peptides, and in particular those produced in the innate immune system, have been shown to have a crucial role in the health and survival of a wide variety of Eukaryotic organisms [4–6,8,9]. Moreover, their failure to limit infection, either at mucosal surfaces or in the phagocytic cells, may be a leading cause of life-threatening infection. For example, *S. aureus* and *Pseudomonas aeruginosa* infections of airway epithelia in the lungs of cystic fibrosis patients are thought to result from their relative insensitivity to human defensins, both as a result of specific resistance factors such as L-PG as well as due to their tolerance to defensins at the unusually high NaCl concentrations found in the cystic fibrosis lung [25,26,37]. Other diseases of humans and animals have also been associated with the ability of specific bacteria to evade the antimicrobial peptides of the innate immune system, such as occurs for *Pseudomonas cepacia* in chronic granulomatous disease or in the pathogenicity of *Salmonella typhimurium* in the intestine [38,39]. Moreover, plants also produce defence peptides with a variety of antimicrobial activities [7,9]; the recent finding that the plant pathogen *Agrobacterium tumefaciens* appears to posess an homologue of *mprF* [26] implies that avoidance of host defence peptides may be essential for the establishment of certain plant diseases and further suggests a practical, ecologically-acceptable approach to their control [8]. Thus, it is important to gather information on defence peptides from a variety of different sources in order to gain a better overview of general, underlying mechanisms in their mode of action and ecological role in nature.

Many antimicrobial peptides, including defence peptides, peptaibols, lantibiotics and bacteriocins, are thought to manifest their respective antibacterial properties in a non-specific manner through the formation of ion-permeable channels or pores in the cytoplasmic membrane of the target organisms [6,10–12,19,40,41]. However, recent studies with several antimicrobial peptides of bacterial origin

Table 1 Comparison of the MIC against *M. luteus*, MHC and HI of Various Synthetic Analogues of Indolicidin

Peptide Sequence	MIC[a]	MHC[b]	HI[c]
ILPWKWPWWPWRR-NH_2	0.4	15.6	39
Ac-ILPWKWPWWPWRR-NH_2	1.6	15.6	10
ILPWKWPWWPWRR-OH	1.6	15.6	10
ILPWKWPWWPWR-NH_2	0.8	15.6	20
ILPWKWPWWPW-NH_2	0.8	31.3	39
ILPWKWPWWP-NH_2	1.6	62.5	39
ILPWKWPWW-NH_2	1.6	62.5	39
ILPWKWPW-NH_2	6.3	125	20
LPWKWPWWPWRR-NH_2	0.2	7.8	39
PWKWPWWPWRR-NH_2	0.8	31.3	39
WKWPWWPWRR-NH_2	0.4	31.3	78
KWPWWPWRR-NH_2	0.4	31.3	78
WPWWPWRR-NH_2	1.6	31.3	20
PWWPWRR-NH_2	3.1	62.5	20
ALPWKWPWWPWRR-NH_2	0.4	15.6	39
I**A**PWKWPWWPWRR-NH_2	0.4	15.6	39
IL**A**WKWPWWPWRR-NH_2	0.4	15.6	39
ILP**A**KWPWWPWRR-NH_2	0.4	15.6	39
ILPW**A**WPWWPWRR-NH_2	0.8	15.6	20
ILPWK**A**PWWPWRR-NH_2	0.8	15.6	20
ILPWKW**A**WWPWRR-NH_2	0.4	15.6	39
ILPWKWP**A**WPWRR-NH_2	0.8	15.6	20
ILPWKWPW**A**PWRR-NH_2	0.8	15.6	20
ILPWKWPWW**A**WRR-NH_2	1.6	15.6	10
ILPWKWPWWP**A**RR-NH_2	1.6	15.6	10
ILPWKWPWWPW**A**R-NH_2	0.4	15.6	39
ILPWKWPWWPWR**A**-NH_2	0.8	15.6	20
ILP**V**K**V**P**VV**P**V**RR-NH_2	>100	>1000	NA[d]
ILP**L**K**L**P**LL**P**L**RR-NH_2	25	62.5	2.5
ILP**I**K**I**P**II**P**I**RR-NH_2	25	62.5	2.5
ILP**F**K**F**P**FF**P**F**RR-NH_2	0.4	125	313
ILP**Y**K**Y**P**YY**P**Y**RR-NH_2	6.3	62.5	10
ILP**S**K**S**P**SS**P**S**RR-NH_2	>100	>1000	NA
ILP**H**K**H**P**HH**P**H**RR-NH_2	>100	>1000	NA
ILP**K**K**K**P**KK**P**K**RR-NH_2	>100	>1000	NA
ILP**E**K**E**P**EE**P**E**RR-NH_2	>100	>1000	NA
ILPW**R**WPWWPWRR-NH_2	0.4	15.6	39
ILPW**O**WPWWPWRR-NH_2	0.4	15.6	39
ILPWKWPWWPW**KK**-NH_2	0.4	31.3	78
ILPWKWPWWPW**UU**-NH_2[e]	25	250	10
IL**G**WKW**G**WW**G**WRR-NH_2	0.4	7.8	20
ILPW**D**WPWWPW**DD**-NH_2	>100	>1000	NA
ILPW**E**WPWWPW**EE**-NH_2	>100	>1000	NA
RRWPWWPWKWPLI-NH_2	0.4	31.3	78
ilpwkwpwwpwrr-NH_2	0.4	15.6	39
rrwpwwpwkwpli-NH_2	0.4	31.3	78
RRFPFFPFKFPLI-NH_2	0.4	250	625
ilpfkfpffpfrr-NH_2	0.4	125	313
rrfpffpfkfpli-NH_2	0.4	250	625

[a] Minimum inhibitory concentration (μM) against *M. luteus* determined in a doubling dilution, agar plate diffusion assay.
[b] Minimum haemolytic concentration (μM) against human erythrocytes determined in a doubling dilution assay.
[c] Haemolytic index is defined as MHC/MIC and represents the fold less haemolytic than inhibitory for a given analogue against human erythrocytes and *M. luteus*, respectively.
[d] NA, not applicable since (at least) one endpoint could not be determined.
[e] U, L-citrulline (Cit).

have suggested that this may not always be a generalized mechanism of action. For example, the lantibiotics nisin, epidermin and gallidermin have been shown to interact with the cell wall biosynthetic precursor lipid II in the target bacterium, which acts to stabilize the pores and has therefore been termed a 'docking molecule' [20,21,42]. In the absence of lipid II, these lantibiotics are still able to form pores, but higher concentrations are required and the pores formed are less stable. Similarly, the bacteriocin pediocin PA-1 has been shown to interact with an as yet unidentified 'receptor-like' molecule in the cell membrane of the target bacterium; the antimicrobial activity of pediocin PA-1 may be blocked by specific synthetic 15-mer peptides derived from a portion of the pediocin PA-1 sequence, suggesting that this region of the peptide associates intimately with a membrane component of the target bacterium [22].

In the present study, we generated retro-, inverso- and retroinverso-analogues of both indolicidin and [Trp4,6,8,9,11Phe]-indolicidin. The retro-analogues (all D-amino acids) of these two peptides displayed essentially the same biological activity as their all L-amino acid-containing counterparts. Therefore, it seems unlikely that indolicidin activity is dependent on the presence of a target-specific cellular component such as appears to be involved in the activity of nisin or pediocin PA-1. This result may reflect the general purpose nature of defence peptides; dependence on a receptor-like molecule in the target increases specificity, an effect that may not be a desirable evolutionary characteristic in a general defence peptide. Further evidence for a non-specific mechanism of action for indolicidin and [Trp4,6,8,9,11Phe]-indolicidin comes from their respective inverso- and retroinverso-analogues. The antibacterial activity of each of these analogues was unaffected, suggesting that a receptor-based mechanism able to distinguish chiral or sequence structure, such as would be expected for a receptor molecule, is unlikely. Interestingly, whilst the retro-analogues were as haemolytic as their all L-amino acid-containing counterparts, inverso- and retroinverso-peptides of both indolicidin and [Trp4,6,8,9,11Phe]-indolicidin may be about 2-fold less haemolytic. This result could be due to the inversion of the relative positions of the termini or to an alteration of the overall dipole moment.

Recently, we have demonstrated that certain pathogenic bacteria, such as *S. aureus*, may modify their surface charge in order to avoid the effects of various cationic antimicrobial peptides [25,26]. In particular, we have identified the protein MprF, a

pathogenic factor rendering strains of *S. aureus* expressing it considerably more resistant to various antimicrobial peptides both *in vitro* and *in vivo*. Moreover, we have shown that MprF is involved in modification of the lipid composition of the plasma membrane; MprF-bearing strains have contain large amounts of lysine-esterified phospholipid, leading to an increased net-positive surface charge. Thus, MprF confers resistance primarily against cationic antimicrobial peptides, since these will be more effectively repelled from the membrane surface by unfavourable electrostatic forces [26]. The present study provides further evidence for the protective role of MprF in *S. aureus* as most of the analogues tested were more active against the MprF-deficient mutant than against the wild-type strain. Furthermore, the overall charge of the peptide analogues plays an important role in determining the biological activity of certain analogues. For example, the analogue [Lys5Ala]-indolicidin showed unaltered activity against the MprF-deficient strain, whilst being inactive against the wild type. Thus, even defence peptides with relatively low overall charge may retain activity against strains in which MprF has been inactivated, suggesting not only that MprF plays a vital role in avoidance of the innate immune system, but also that antimicrobial therapy targeting MprF activity could render *S. aureus* susceptible to host defence peptides and might therefore provide an effective interventive therapeutic measure.

It has previously been shown that an increase in the overall charge of indolicidin may improve its biological activity, particularly against pathogens such as *S. aureus* [17,29]. However, charge is not the sole factor determining the biological activity of a given analogue against MprF-bearing or MprF-deficient strains of *S. aureus*. Whilst it might be expected that negatively-charged analogues (e.g. [Trp4,6,8,9,1Glu]-, [Lys5/Arg12,13Asp]- and [Lys5/Arg12,13Asp]-indolicidin) would be biologically inactive, other analogues including several in which no change to overall charge had occurred or analogues such as [Trp4,6,8,9,11Lys]-indolicidin, where the overall basic nature of the peptide is markedly increased were also inactive against all strains tested. Moreover, the replacement of Trp with the aromatic, imino-residue histidine also abolished antimicrobial activity, suggesting that the position and/or distribution of the positively charged residues is of overriding importance. This is further supported by our observation that the nature of the positively charged residues plays little role in the antimicrobial activity; replacement of the guanidino amino acid arginine with the aliphatic amine lysine, replacement of lysine with arginine or the replacement of lysine with ornithine (shorter aliphatic amine side chain) had minimal effect on biological activity, whilst the analogue [Arg12, 13Cit]-indolicidin, in which Arg residues were replaced with the arginine guanidinamide analogue citrulline, showed considerably reduced antimicrobial activity against both MprF-bearing and MprF-deficient strains.

Previous studies have shown that the terminal functions of indolicidin play an important role in determining both antibacterial and haemolytic activity [17,29]. For example, a carboxymethylated analogue showed improved antibacterial activity and a concomitantly reduced haemolytic character. The present study further suggests that the terminal functions of indolicidin are of particular importance in determining the peptides biological activity. Increasing *C*-terminal hydrophilicity by the introduction of a carboxy function in place of the carboxamide found in indolicidin, or acylation of the *N*-terminal primary amine by acetylation, both resulted in considerably reduced antimicrobial activity. Interestingly, the acetylated analogue, Ac-indolicidin, also showed a 2-fold reduction in biological activity against the MprF-deficient strain, probably because of the overall reduction in its charge. Furthermore, both terminal modifications have a greater effect on the anti-*E. coli* activity of the peptide, suggesting that the terminal groups may play an even greater role in the anti-Gram-negative biological activity of this peptide. In addition, the analogues Ac-indolicidin and indolicidin-OH retain the same level of haemolytic activity as indolicidin (i.e. the analogues have a lower haemolytic index), suggesting both that the terminal functional groups play little role in the haemolytic activity of the peptide and that indolicidin may have evolved the *C*-terminal carboxamide as a means to increase its relative antibacterial activity without increasing its haemolytic character.

The role of the proline residues to the secondary structure of indolicidin has been controversial [17,33]. However, in the present study we have also shown that the exchange of proline residues may have an effect on biological activity. Whilst the single amino acid exchange [Pro3Ala]-indolicidin had little affect on either the antibacterial or haemolytic activities, [Pro7Ala]-indolicidin showed no change in anti-staphylococcal activity, but was 2-fold less active against *E. coli*, while [Pro10Ala]-indolicidin

was less active against both the Gram-positive and the Gram-negative pathogens. Moreover, the multiple exchange [Pro3,7,10Gly]-indolicidin displayed only minimally altered activity against *E. coli* but was approximately 2-fold more haemolytic than indolicidin. Together, these results demonstrate that it is possible to alter the relative activity of indolicidin against different pathogens or haemocytes and that (at least) the proline residues are important in this respect. Moreover, exchanges of Pro to Gly should have little effect on overall secondary structure, although they may reduce the overall hydrophilicity of the peptide. Thus, it appears that the secondary structure is probably an important determinant of the relative activity against different pathogens, whilst the physicochemical nature of the peptide may be more relevant in determining the haemolytic activity of a given analogue.

Indolicidin is a relatively symmetric molecule, particularly in the region Lys5 to Arg12. Moreover, since the analogues [Lys5Arg]-, [Lys5Orn]- and [Arg12Lys]-indolicidin displayed similar activities to indolicidin and [Trp4,6,8,9,11Phe]-indolicidin, a central 'core' region can be defined as XOPOOPOX, where X is a basic amino acid and O is either Trp or Phe. Interestingly, this putative core structure is a palindrome or inverted repeat; thus, inverso-peptides will contain the same sequence, perhaps explaining the observation that inverso-analogues of both indolicidin and [Trp4,6,8,9,11Phe]-indolicidin display essentially unaltered antibacterial activity. Furthermore, the analogues [Lys5Ala]- and [Arg12Ala]-indolicidin displayed significantly reduced activities, whilst deletions in the first 4 amino acids at the *N*-terminus had little effect on activity (or may even improve activity), suggesting that the 'core' region is flanked by the essential basic amino acids but that the *N*-terminal tail is neither necessary for biological activity against the organisms tested here (although it may well influence the biological activity against other, untested microorganisms), nor for haemolytic activity. Interestingly, it has recently been shown by analysis of the NMR structure of indolicidin in association with phospholipid micelles, that this region is involved in membrane penetration, suggesting that these two flanking basic amino acids may be located at the membrane/aqueous solution interface during pore formation [34]. While the 'core' palindrome of indolicidin is not the only determinant of indolicidin biological activity, it may provide a structural basis or scaffold for the development of related mimetic structures. To this end, we are currently investigating non-peptidyl substituents and bonds for the replacement of the hydrophobic aromatic residues Trp and Phe.

Overall, the study of structure function relationships in indolicidin reveals several interesting features. Firstly, the results from the deletion analogues, together with the undisrupted activity of the inverso-analogues suggest that indolicidin contains a 'core' region essential for biological activity, the flanking regions of which may be more involved in antimicrobial specificity. Secondly, it is possible not only to alter the relative haemolytic activity of indolicidin but also to alter relative antimicrobial activity (e.g. anti-Gram-positive or anti-Gram-negative activities) by the preparation of appropriate analogues. Finally, the chiral nature of the amino acids in indolicidin is not a determinant of biological activity, suggesting that the mechanism of action is not receptor or docking molecule mediated. Moreover, since D-amino acid-containing antimicrobial peptides (e.g. retro-cecropin B) have been shown to be considerably more stable to proteolytic degradation [43,44], retro-[trp4,6,8,9,11phe]-indolicidin may provide an effective antimicrobial agent with low haemolytic activity, at least as a lead structure for the development of related mimetic compounds. Clearly, the results of the present study suggest that further development of this peptide is warranted.

Acknowledgements

This research was supported by the *Deutsche Forschungsgemeinschaft* (DFG), *Sonderforschungsbereich* (SFB) 323. We thank Dr Holger Eickhoff and Dr Martin Welter (EMC microcollections GmbH, Tübingen) for valuable discussions and Graeme Nicholson (Institut für Organische Chemie, Universität Tübingen) for expert chiral-phase analyses.

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