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Antimicrobial Activity of Short Arginine- and Tryptophan-rich Peptides

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Abstract: Highly antimicrobial active arginine- and tryptophan-rich peptides were synthesized ranging in size from 11 to five amino acid residues in order to elucidate the main structural requirement for such short antimicrobial peptides. The amino acid sequences of the peptides were based on previous studies of longer bovine and murine lactoferricin derivatives. Most of the peptides showed strong inhibitory action against the Gram-negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa*, and the Gram-positive bacterium *Staphylococcus aureus*. For the most active derivatives, the minimal inhibitory concentration values observed for the Gram-negative bacteria were 5 μg/ml (3.5 μM), whereas it was 2.5 μg/ml (1.5 μM) for the Gram-positive bacterium. It was essential for the antimicrobial activity that the peptides contained a minimum of three tryptophan and three arginine residues, and carried a free *N*-terminal amino group and an amidated *C*-terminal end. Furthermore, a minimum sequence size of seven amino acid residues was required for a high antimicrobial activity against *Pseudomonas aeruginosa*. The insertion of additional arginine and tryptophan residues into the peptides resulted only in small variations in the antimicrobial activity, whereas replacement of a tryptophan residue with tyrosine in the hepta- and hexapeptides resulted in reduced antimicrobial activity, especially against the Gram-negative bacteria. The peptides were non-haemolytic, making them highly potent as prospective antibiotic agents.

Keywords: arginine; lactoferricin; minimal inhibitory concentration; short antimicrobial peptides; tryptophan

INTRODUCTION

During the past 30 years or so, no new chemical classes of systemically active antibiotics have been introduced into the clinic [1]. The pharmaceutical industry has mainly focused on semisynthetic modifications of existing antibiotics in order to circumvent resistance among pathogenic bacteria. However, an increasing number of multiresistant bacteria are causing serious problems, especially in hospital environments. Methicillin resistant *Staphylococcus aureus* (MRSA) strains are resistant to all β-lactam antibiotics because of the production of a penicillin binding protein encoded by the *mecA* gene with much lower affinity for β-lactams [2]. Vancomycin is among the few choices of clinical treatment against MRSA. However, enterococcal strains that are resistant to vancomycin have been known for some time, and transfer of resistance to MRSA is feared [3]. It has already been shown in the laboratory that *S. aureus* can acquire vancomycin resistance from enterococci [4].

For future treatment of serious infections a new class of systemically and topically active antibiotics

Abbreviations: ATCC, American Type Culture Collection; EC_{50}, concentration of peptide required for 50% haemolysis; MBC, minimal bactericidal concentration; MIC, minimal inhibitory concentration; RBC, human red blood cells.

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may be developed from naturally occurring peptides. In nature, these peptides constitute an important part of the innate immunity of animals and insects [5,6], as exemplified by the cecropins [7], defensins [8–10] and magainins [11]. This first line of defence is characterized by amphipathic peptides with a net positive charge and consisting of less than 60 amino acids [12]. Their mechanism of inhibiting bacterial growth is by an unspecific binding to the negatively charged phospholipids of the bacterial cell membrane, followed by disruption of the membrane integrity at a critical peptide concentration (see reviews [12–16]). Some antimicrobial peptides are also capable of accessing the cytoplasm without disrupting the bacterial cell membrane, and inhibitions of DNA replication and transcription are suggested as possible targets for these peptides [15,17–20].

The relatively large size of naturally occurring antimicrobial peptides and their sometimes complex disulphide pattern, as found in the defensins [21], often makes them difficult and expensive to synthesize chemically. However, shorter and more cost efficient derivatives of naturally occurring antimicrobial peptides can be prepared. A 25 residue peptide, encompassing residues 17 to 41 (LFB 17–41) of the mature bovine lactoferrin protein, was reported in 1991/1992 to display antimicrobial activity [22,23]. This protein fragment was named bovine lactoferricin, and in 1996 it was demonstrated that an undecapeptide, encompassing residues 20 to 30 (LFB 20–30) of the bovine lactoferrin protein, displays similar antimicrobial activity as the 25 residue fragment [24]. The antimicrobial activity of LFB 20–30 was further increased by a simple modification involving the replacement of all the lysine residues with arginine [24]. In a recent paper we have shown that the activity of a homologous undecapeptide, encompassing residues 17 to 27 (LFB 17–27) of bovine lactoferrin, can be substantially improved by the incorporation of tryptophan residues [25]. Even shorter derivatives of lactoferricins display antimicrobial activity, and the active centre of bovine lactoferricin is reported to constitute a hexapeptide corresponding to residues 20 to 25 (LFB 20–25) [26]. *C*-terminal amidation of this peptide results in a major improvement of the antimicrobial activity against *E. coli* and *S. aureus* [26]. It is also noteworthy that a series of hexapeptides that resembles the active centre of bovine lactoferricin was prepared in a study using a synthetic-combinatorial library approach for finding new antimicrobial lead compounds [27].

The development of short antimicrobial peptides serves a number of advantages by being both easy to synthesize chemically and simple to purify. A limited amount of time and solvents are needed, which is advantageous both from an economical and environmental point of view. By carefully investigating the sequences of naturally occurring antimicrobial peptides, shorter derivatives with similar or even enhanced biological activities can be prepared. In this study we have exemplified this by preparing a series of modified short peptides, ranging in size from 11 to five amino acid residues, which were based on previous studies of highly active pentadeca- and undecaderivatives of bovine and murine lactoferricins [24,25,28,29]. In accordance with these previous investigations, all peptides prepared were rich in arginine and aromatic amino acids, and contained an amidated *C*-terminal end. The present peptides displayed antimicrobial activities against *E. coli*, *P. aeruginosa* and *S. aureus* and were generally non-haemolytic.

METHODS

Preparation of Peptides

All the peptides were synthesized on an automatic 9050 *Plus* PepSynthesizer (Milligen, Milford, MA, USA) using a solid-phase strategy with Fmoc-protection, as described previously [25]. All the peptides were analysed by reversed phase-high performance liquid chromatography (analytical C18-column, Delta-Pak™ C18, 100 Å, 5 μM), 3.9 × 150 mm, Waters Corporation) and positive ion electrospray ionization mass spectrometry (VG Quattro quadrupole mass spectrometer, VG Instruments Inc., Altrincham, UK). The purity of all peptides was found to be >98%.

Antimicrobial Activity

A standard microdilution technique with an inoculum of 2×10^6 cfu/ml was used to determine the antimicrobial activity against the test bacteria *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATCC 25923, as described previously [25]. All the peptides were tested in triplets using the following concentration series: 200, 150, 100, 80, 75, 50, 25, 20, 15, 10, 7.5, 5, 2.5, 1 and 0.5 μg/ml.

Haemolytic Activity

The haemolytic activity of the peptides was determined using human red blood cells (RBC) isolated from heparinized blood and tested according to the following concentration series: 1000, 500, 100, 50, 10 and 1 μg/ml [25,30]. Phosphate buffered saline and 1% Triton X-100 (Sigma, St. Louis, MO, USA) were used as controls for zero and 100% haemolysis, respectively.

RESULTS AND DISCUSSION

Antimicrobial Activity

The most active peptide characterized previously by us is the undecapeptide Undeca 9 (Table 1), which was based on a modified pentadecaderivative of murine lactoferricin [25]. In order to investigate the main structural requirement for short antimicrobial peptides, we first chose to truncate Undeca 9 from the *C*-terminal end and to vary the content of arginine and aromatic residues in positions 1, 4, 7 and 8. Thus, the first series of peptides prepared ranged in size from 11 to eight amino acid residues and were all rich in arginine and aromatic amino acids, which are known to be important for the antimicrobial activity of lactoferricins and shorter peptides derived thereof (Table 1. Top section: peptides Deca 1 to Octa 2) [24,25,28,29]. Compared with Undeca 9, a similar or even enhanced antimicrobial activity against the three test bacteria was observed for the resulting peptides. They all displayed MIC values between 2.5 μg/ml (1.5 μM) and 10 μg/ml (7.0 μM) (Table 1). As observed previously for our series of undecapeptides [25], a higher antimicrobial activity was observed against the Gram-positive than against the Gram-negative test bacteria. Hence, the most active peptides against *S. aureus* displayed MIC values of 2.5 μg/ml (1.5 μM), whereas the most active peptides against *E. coli* and *P. aeruginosa*, displayed MIC values of 5 μg/ml (3.5 μM).

Based on these results, we prepared a second series of homologous peptides that ranged in size from seven to five amino acid residues (Table 1. Bottom section: peptides Hepta 1 to Penta 2). Hexa 1 contained an alternating sequence of three arginine and three tryptophan residues, and despite its short size, was one of the most antimicrobial active peptides against *E. coli* with a MIC value of 5 μg/ml (4.8 μM). The heptapeptides, Hepta 1 and Hepta 2, and the hexapeptides, Hexa 1 and Hexa 2, retained the antimicrobial activity against *E. coli* and *S. aureus* that was displayed by the longer peptides. However, except for Hepta 1, all of the shorter peptides prepared showed a reduced antimicrobial activity against *P. aeruginosa* (Table 1).

It is noteworthy that for peptides shorter than eight amino acid residues, replacement of a tryptophan residue with a tyrosine residue resulted in reduced antimicrobial activity. This effect was observed when comparing the antimicrobial activities of Hepta 1 with Hepta 2, and when comparing the activities of the hexapeptides, Hexa 1 with Hexa 4, and Hexa 2 with Hexa 3. The reduction in antimicrobial activity was most severe against *P. aeruginosa*, but only observed against *S. aureus* for the hexapeptides. As noted previously, the smaller size of the side chain of tyrosine compared with tryptophan may be the reason for this negative effect [25].

The present peptides carried an amidated *C*-terminal end, which we have shown previously to be important for a high antimicrobial activity of undecapeptides [25], and they had a free *N*-terminal amino group which we also believe is of importance. It is clear from the present study that the main structural requirement for a high antimicrobial activity of such short arginine and tryptophan rich peptides is that they contain a minimum of three residues of each amino acid. Accordingly, shorter peptides that do not fulfil the latter requirement, such as the present pentapeptides, displayed a lower antimicrobial activity. However, when this requirement was fulfilled, as shown for the present peptides that contained more than six residues and our previous series of undecapeptides [25], additional arginine or aromatic residues only resulted in small changes in the antimicrobial activity of the peptides. In the case of *P. aeruginosa*, a sequence length of at least seven amino acid residues was required for a high antimicrobial activity.

Comparisons with Other Short Antimicrobial Peptides

Although the activity of the most active pentapeptide, Penta 1, was lower than the activity of most peptides prepared in this study, it still displayed activity in the same range as the most active pentadecapeptide containing naturally encoded amino acids, LFM R1,9 W8 Y13 (RKCLRWQWRMRKYGG), reported by us [29]. For comparison, this three-fold larger peptide displays MIC values against *E. coli*, *P. aeruginosa* and *S. aureus* of 20 μg/ml (10 μM),

Table 1 Antimicrobial and Haemolytic Activities of Short Arginine- and Tryptophan-rich Peptides Ranging in size from 11 to five Amino Acid Residues

Name of peptide	Molecular mass[a]		Amino acid sequence (single letter code)[b]											MIC[c] *E. coli*		MIC[c] *P. aeruginosa*		MIC[c] *S. aureus*		Haemolysis[d] EC_{50}
	Obs.	(Calc.)	1	2	3	4	5	6	7	8	9	10	11							
Undeca 9[f]	1722.0	(1721.0)	R	R	W	Y	R	W	A	W	R	M	R-NH_2	10	(5.8)	7.5	(4.4)	5	(2.9)	N.h.[e]
Deca 1	1620.9	(1620.0)	Y	R	W	W	R	W	A	R	R	W-NH_2		10	(6.2)	7.5	(4.6)	2.5	(1.5)	720 (444)
Nona 1	1434.7	(1433.9)	Y	R	W	A	R	W	W	R	R-NH_2			10	(7.0)	5	(3.5)	5	(3.5)	N.h.
Octa 1	1363.6	(1362.6)	R	R	W	Y	R	W	W	R-NH_2				5	(3.7)	5	(3.7)	2.5	(1.8)	N.h.
Octa 2	1387.6	(1386.4)	R	R	W	W	R	W	W	R-NH_2				7.5	(5.4)	5	(3.6)	2.5	(1.8)	N.h.
Hepta 1	1231.4	(1230.2)		R	W	W	R	W	W	R-NH_2				10	(8.1)	7.5	(6.1)	2.5	(2.0)	920 (748)
Hepta 2	1207.4	(1207.3)		R	W	W	R	Y	W	R-NH_2				10	(8.3)	20	(17)	2.5	(2.1)	N.h.
Hexa 1	1044.2	(1043.8)		R	W	R	W	R	W-NH_2					5	(4.8)	20	(19)	5	(4.8)	N.h.
Hexa 2	1075.2	(1074.2)		W	W	R	W	R	W-NH_2					10	(9.3)	20	(19)	5	(4.7)	590 (549)
Hexa 3	1051.2	(1050.8)		Y	W	R	W	R	W-NH_2					20	(19)	50	(48)	10	(9.5)	600 (571)
Hexa 4	1021.2	(1020.9)		R	W	R	Y	R	W-NH_2					50	(49)	80	(78)	10	(9.8)	N.h.
Penta 1	888.0	(887.5)			W	R	W	R	W-NH_2					15	(17)	50	(56)	10	(11)	N.h.
Penta 2	858.0	(857.5)		R	W	R	W	R-NH_2						200	(233)	50	(58)	50	(58)	N.h.

[a] Observed (calculated) molecular mass.
[b] The $-NH_2$ group denotes peptides with an amidated *C*-terminal carboxylic acid group.
[c] MIC: minimal inhibitory concentration in μg/ml and (μM).
[d] EC_{50}: concentration in μg/ml and (μM) required for 50% haemolysis.
[e] N.h.: no haemolytic activity within the concentration range tested, i.e. up to 1000 μg/ml.
[f] Strøm MB, Rekdal Ø, Svendsen JS [25].

200 μg/ml (98 μM) and 25 μg/ml (12 μM), respectively ([29] and MB Strøm unpublished results). Most of these shorter arginine and tryptophan rich peptides also displayed a higher antimicrobial activity than magainin 2, which when tested according to our method displays MIC values against *E. coli* and *S. aureus* of 20 μg/ml (8 μM) and >100 μg/ml (>40 μM), respectively (JS Svendsen unpublished results).

Both the highly active core peptide of bovine lactoferricin, RRWQWR-NH_2 [26], and a series of hexapeptides (Ac-RRWWCX-NH_2) isolated from a library consisting of thousands of peptides [27], contain the same three *N*-terminal amino acids. This *N*-terminal RRW-motif was also present in the peptides Undeca 9, Octa 1 and Octa 2 of this study and could, therefore, contribute to their activity against bacteria. However, as shown for the present Deca 1, Nona 1, Hepta 1 and Hexa 1 peptides, replacement, or even elimination of the *N*-terminal arginine residue in this motif resulted in peptides that displayed similar or even better antimicrobial activities. Thus, the order of the amino acids in such short peptides is not essential for their antimicrobial activity. More important is the total amount and type of cationic and lipophilic residues used in short peptides, in which we have found that arginine and tryptophan are superior among naturally encoded amino acids.

It is difficult to compare the antimicrobial activities of peptides from different studies since different bacterial strains and experimental procedures are used. For the most active peptide determined from a library of hexapeptides, Ac-RRWWCF-NH_2, the necessary concentration for 50% inhibition of bacterial growth (IC_{50}) is in the range 20–21 μg/ml, 42–45 μg/ml and 10–14 μg/ml for *E. coli*, *P. aeruginosa* and *S. aureus*, respectively [27]. It is noteworthy that this peptide, which is seemingly less active than our Hexa 1 peptide, does not fulfil the main structural requirement for antimicrobial activity of short arginine- and tryptophan-rich peptides stated above. Firstly, the peptide Ac-RRWWCF-NH_2 has a lower net charge than Hexa 1 since it only contains two arginine residues and has an acetylated *N*-terminal amino group. Secondly, instead of a third tryptophan residue, it contains a phenylalanine residue which is less efficient than tryptophan in short antimicrobial peptides due to its smaller size [25,31]. Corresponding peptides with either a *C*-terminal arginine residue or a tryptophan residue are even less active than Ac-RRWWCF-NH_2 [27].

Mechanism of Action

A high amount of cationic residues is important to ensure a strong electrostatic interaction between the peptides and the negatively charged phospholipids of the bacterial cell membrane, and the outer lipopolysaccharide layer of Gram-negative bacteria [12]. In this study arginine was chosen as the cationic residue since its side chain can interact both electrostatically and by hydrogen bond formation with the negatively charged surface of bacteria. As lipophilic residues, aromatic amino acids were used since both tryptophan and tyrosine are essential for the antimicrobial activity of pentadeca- and undecaderivatives of lactoferricins [25,28,29]. This effect is attributed to their preference for the water-phospholipid interface region when incorporated into biological membranes [32,33]. The size of the aromatic residues is essential in the disruption of membrane integrity, and can explain the reduction in activity observed when tryptophan was replaced by tyrosine in the present peptides [25]. A similar correlation between antimicrobial activity and the bulkiness of lipophilic residues has been demonstrated for pentadecaderivatives of bovine lactoferricin containing unnatural aromatic amino acids [34,35]. Thus, according to the two-state model of Huang [36], the minimal structural requirement of three tryptophan residues deduced in the present study ensured a maximal thinning of the membrane in a certain radius around the peptides, followed by disruption of the membrane integrity at a critical peptide concentration.

Haemolytic Activity

Haemolytic activity was used as a coarse measurement of the toxicity of the peptides. The short peptides prepared in this study were essentially non-haemolytic, i.e. showed EC_{50} values above 590 μg/ml (Table 1). Detectable haemolytic activity was only observed for some of the peptides that contained four or more aromatic residues, and except for Hepta 2, had a higher content of aromatic residues than arginine residues. A similar correlation was also observed between haemolytic activity and a high amount of aromatic residues versus cationic residues for a series of undecapeptides reported recently [25]. Thus, there is a strong correlation between the number of aromatic residues, especially tryptophan, and the haemolytic activity of antimicrobial peptides. Fortunately, peptides containing only three tryptophan residues are highly

antimicrobial, but non-toxic. The low haemolytic activity of the present peptides was therefore an effect of their short size and relatively low hydrophobicity. Apparently, this rendered them highly selective for bacteria, which contain a higher proportion of anionic phospholipids compared with zwitterionic phospholipids in RBC [37].

CONCLUSION

The combination of three arginine and three tryptophan residues, and an amidated *C*-terminal end, constitutes a highly efficient motif in short antimicrobial peptides. The order of the amino acids in peptides fulfilling this main structural requirement was not essential for the antimicrobial activity, and except against *P. aeruginosa*, additional arginine or aromatic residues did not result in major improvement of the activity of the peptides. The arginine residues ensured a strong electrostatic interaction between the peptides and the negatively charged bacterial surface, whereas the large size of the Trp residues ensured an efficient disruption of the membrane integrity. The high antimicrobial activity and low toxicity of such short peptides makes them highly potent as novel agents for both topical and systemic treatment of bacterial infections.

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