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Letters

The Pharmacophore of Short Cationic Antibacterial Peptides

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Abstract: Cationic antibacterial peptides have been proclaimed as new drugs against multiresistant bacteria. Their limited success so far is partially due to the size of the peptides, which gives rise to unresolved issues regarding administration, bioavailability, metabolic stability, and immunogenicity. We have systematically investigated the minimum antibacterial motif of cationic antibacterial peptides regarding charge and lipophilicity/bulk and found that the pharmacophore was surprisingly small, opening the opportunity for development of short antibacterial peptides for systemic use.

Introduction. Since cationic antibacterial peptides were discovered in the hemolymph of insects in the late 1970s,[1] they have been found in all animal kingdoms and are now recognized as key elements of innate immunity.[2] Cationic antibacterial peptides are defined as peptides containing a net excess of positively charged residues, approximately 50% hydrophobic residues and a size ranging from 12 to 50 residues.[3] Most cationic antibacterial peptides have a propensity to fold into amphiphilic structures where the hydrophobic residues are concentrated on one face of the molecule whereas the cationic groups are located on the opposite face. The importance of the cationic/hydrophobic segregation has been demonstrated by the potent antibacterial properties found in amphiphilic helices comprising β-peptides.[4] The amphiphilicity of the cationic antibacterial peptides is a key requisite for the peptides to interact with their primary target, the bacterial cell membrane.[5]

The unusual primary target of cationic antibacterial peptides in contrast to antibiotics in regular clinical use renders the peptides effective against bacteria that have developed cross-resistance against conventional antibiotics. Furthermore, membrane-active peptides show low propensity for development of resistance because of their unique mode of action, and antibiotic peptides have been heralded as new antibiotic drug candidates targeting multiresistant pathogens. Despite these attractive properties, cationic antibacterial peptides have not captured the interest of large pharmaceutical companies. This may be due to the fact that most antibiotics in clinical use are small, stable, nontoxic, and inexpensive,[6] while antibiotic peptides have unresolved issues regarding toxicity and stability. Furthermore, cationic antibacterial peptides are usually so large that production cost as well as a potential lack of systemic applicability represents additional concern. It is thus important to develop smaller antibacterial peptides (with less than six residues) in order to alleviate the concern of production cost and reduce potential immunogenic responses. Peptides of this size range should be fairly easy to construct with adequate metabolic stability, thus allowing for systemic application of cationic antibacterial peptides. There are only a few examples of antibacterial peptides of this size in the literature, most notably N-acetylated hexapeptides and *N*-Fmoc tetrapeptides identified by deconvolution of combinatorial libraries[7,8] as well as the six-residue antibiotic core peptide of bovine lactoferricin.[9] We have recently prepared an antibiotic pentapeptide based on the concept that cationic charge (Ch) and bulk and lipophilicity (B) are major factors determining antibacterial activity in short peptides.[10] We have in the present work undertaken a systematic study into the minimum requirement of the charge and bulky/lipophilic properties in antibacterial peptides against the Gram-negative rod *Escherichia coli* and the Gram-positive cocci *Staphylococcus aureus* in order to define the pharmacophore of cationic antibacterial peptides. The effect of the peptides on multiresistant staphylococci as well as toxicity against erythrocytes is also addressed.

Results. Experimental Design. Several research groups, including ours, have previously shown that the

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Table 1. Antibacterial Activity (MIC) in μg/mL against *E. coli* and *S. aureus* of Peptide Amides

peptide sequence	molecular mass calcd	molecular mass obsd	MIC *E. coli*	MIC *S. aureus*
WRWRWR-NH_2	1044.2	1044.0	10	7.5
RWRWRW-NH_2	1044.2	1044.0	5	5
RRRWWW-NH_2	1044.2	1044.0	5	5
RWWWRR-NH_2	1044.2	1044.0	25	5
WWRRRW-NH_2	1044.2	1044.0	25	10
WRWRW-NH_2	888.0	887.5	15	10
RWRWR-NH_2	858.0	857.5	200	25
WRYRW-NH_2	865.0	864.8	100	50
WRWRY-NH_2	865.0	864.8	100	50
WRWR-NH_2	701.8	701.5	>200	200
WRRW-NH_2	701.8	701.5	>200	200
RWWR-NH_2	701.8	701.5	>200	100
WRW-NH_2	545.6	545.3	>200	100
RWR-NH_2	515.6	515.5	>200	>200

antibacterial activity of small peptides is mainly governed by charge and lipophilic bulk and that these properties should be balanced in order to avoid eukaryotic toxicity.[10−12] The present study was designed to identify the minimal motif, or pharmacophore, for antibacterial activity in cationic peptides and to identify the tolerable variation within the motif. To simplify matters, arginine was chosen to represent the charged moiety because it is charged under all physiological conditions, whereas tryptophan was chosen to represent the properties of lipophilic bulk. The first series of peptides was designed with a balanced content of charged and bulky/lipophilic groups. The majority of the peptides were made with an unmodified amino terminal, while the C terminal was blocked as an amide. In certain peptides, the slightly smaller tyrosine residue replaced a tryptophan residue. The antibacterial activities of the peptides designed are compiled in Table 1.

Table 1 shows that the hexapeptides were effective antibacterial agents irrespective of the order of the amino acids. Against *S. aureus* there was virtually no difference in activity between all sequence variations ranging from alternating sequences to the peptide where all the cationic residues were located at the N terminus and the bulky and lipophilic residues were located at the C terminus. A similar trend was observed against *E. coli* with the exception of the peptides where a charged or lipophilic residue is added to the end of a lipophilic or charged segment, RWWWRR-NH_2 or WWRRRW-NH_2, respectively, which showed markedly lower activity. For the pentapeptides, the results showed that the peptides with an excess of tryptophan displayed the highest activity and that tyrosine was less effective in providing bulk and lipophilicity than tryptophan, thus lowering antibacterial activity. The results for the pentapeptides also showed that the antibacterial effect against *E. coli* was more sensitive for inadequate bulk and lipophilicity in the peptides, whereas *S. aureus* maintained its susceptibility for these peptides although at a higher minimal inhibitory concentration (MIC) level. None of the tetra- or tripeptides were active against *E. coli*, but it is interesting to note that a tripeptide (WRW-NH_2) displayed activity against *S. aureus*, albeit weakly with an MIC value of 100 μg/mL.

On the basis of the results from the initial study, a series of tetra-, tri-, and dipeptide benzyl or methyl ester derivatives were prepared. In this second series of peptides, D-amino acids were also included. The results from the peptide ester derivatives are compiled in Table 2.

Table 2. Antibacterial Activity (MIC) in μg/mL against *E. coli*, *S. aureus*, MRSA, and MRSE of Peptide Esters

peptide sequence[a]	molecular mass calcd	molecular mass obsd	MIC *E. coli*	MIC *S. aureus*	MIC MRSA[b]	MIC MRSE[c]
RWWR-OMe	716.8	717.4	>200	75	50	15
RWRW-OBzl	792.9	793.4	50	2.5	2.5	2.5
RWrw-OBzl	792.9	793.4	75	5	2.5	2.5
WRWR-OMe	530.6	531.4	>200	>200	100	75
WWR-OMe	560.6	561.4	>200	>200	200	100
WRW-OBzl	636.7	637.4	75	5	5	5
wrw-OBzl	636.7	637.3	75	5	5	5
wRW-OBzl	636.7	637.4	75	20	20	10
WR-OMe	374.4	375.2	>200	>200	>200	>200
WR-OBzl	450.5	451.2	>200	200	75	75
RW-OBzl	450.5	451.1	>200	15	15	10
Rw-OBzl	450.5	451.3	>200	50	50	25
rW-OBzl	450.5	451.4	>200	25	25	25
rw-OBzl	450.5	451.1	>200	50	50	20
KW-OBzl	422.5	423.3	>200	50	25	25
Kw-OBzl	422.5	423.3	>200	75	50	25
kW-OBzl	422.5	423.3	>200	75	50	25
RF-OBzl	411.5	412.3	>200	200	200	75
FR-OBzl	411.5	412.2	>200	>200	200	200
KF-OBzl	383.4	384.4	>200	>200	>200	200

[a] Capital letters denote L-amino acids, and lower case letters denote D-amino acids. [b] MRSA: methicillin-resistant *S. aureus* (ATCC 33591). [c] MRSE: methicillin-resistant *S. epidermidis* (ATCC 27626).

All the dipeptides were inactive against *E. coli* at the concentration range tested, but in contrast to the peptide amides of the first series, several of the tetra- and tripeptide esters displayed antibacterial activity. The active peptides, all benzyl esters, contained at least two tryptophan residues. It should be noted that having two tryptophan residues alone was insufficient for activity against *E. coli*; hence, the methyl ester derivatives were all inactive against this strain.

S. aureus was more susceptible than *E. coli* to the peptide esters, and the methicillin-resistant strains MRSA and MRSE were generally even more sensitive than the nonresistant *S. aureus*. Several tetra- and tripeptides were highly active, displaying MIC values below 10 μg/mL; moreover, antibacterial activity could even be observed for dipeptides. The minimal requirement for anti-staphylococcal activity was a net charge of +2 and the presence of at least two bulky/lipophilic moieties. For the shortest peptides, the latter requirement was simply fulfilled by the presence of one tryptophan residue and a C-terminal benzyl ester. The combination of two tryptophan residues and a C-terminal methyl ester derivative (e.g., WWR-OMe) gave rise to slightly less efficient anti-staphylococcal peptides. The results also showed that both the replacement of arginine by lysine and the replacement of tryptophan by phenylalanine led to less active peptides. Cationic antibacterial peptides containing D-amino acids may display higher antibacterial activity because of increased resistance against proteases. However, the incorporation of a single D-amino acid into the short peptides shown in Table 2 resulted in decreased antibacterial activity compared to the all L-analogues; thus, the potential enzymatic stability provided by D-amino

Table 3. Antibacterial Activity (MIC) in μg/mL against *E. coli* and *S. aureus* of N-Modified Peptide Esters

	molecular mass		MIC	
peptide sequence[a]	calcd	obsd	*E. coli*	*S. aureus*
Ind-RW-OBzl	607.7	608.3	150	20
Chx-RW-OBzl	560.7	561.5	200	25
Ind-WR-OMe	531.6	532.6	200	200
Chx-WR-OMe	484.6	485.3	>200	>200

[a] Ind: 3-indolylacetamide. Chx: cyclohexylcarboxamide.

acids did not offer any advantage in peptides of four amino acids or less.

In a third series of cationic antibacterial peptides, dipeptide esters where the N-terminal residue was modified as a 3-indolylacetamide (Ind) or a cyclohexylcarboxamide (Chx) were prepared. These peptides can be regarded as tripeptides containing an N-terminal desamino residue, e.g., 3-indolylacetamide represented an N-terminal desaminotryptophan. The N-terminal-modified peptides were only slightly less active than their "normal" counterparts; the peptide Ind-RW-OBzl was only one titer step less active than WRW-OBzl (Table 3). The Chx modification yielded peptides that were slightly less active than the Ind derivatives.

None of the peptides in the present study displayed hemolytic activity below 500 μg/mL.

Discussion. The results presented showed that high antibacterial activity was obtained with surprisingly small peptides containing charged (Ch) as well as bulky and lipophilic (B) moieties. The charged moieties consisted of either the side chains of arginine or lysine, where the guanidinium group of arginine was preferred, or the N-terminal amino group. Indole, phenol, or phenyl groups represented the units of bulk. Although no quantitative relationship was determined, the clear trend was that the larger indole moiety produced more effective peptides than corresponding peptides containing the smaller phenol or phenyl groups. These findings were in accordance with results obtained for the 15-residue cationic antibacterial peptides based on the bovine lactoferricin sequence.[11,13−16] The concept of bulky units also covered N- and C-terminal modifications. Thus, the methyl ester peptide derivatives were only slightly more active than the peptide amides and did not constitute a bulky unit, whereas the benzyl ester conferred the properties of bulk/lipophilicity, resulting in peptide esters that were more active than both the amide or methyl ester analogues.

The results also revealed that the order of the amino acids in the peptides was less important for antibacterial activity than the net content of bulky and lipophilic groups. However, the shorter peptides with the bulky and lipophilic groups nearby tended to be slightly more active; RW-OBzl was more active than WR-OBzl, and RF-OBzl was more active than FR-OBzl.

The minimum content of charge and of bulk and lipophilicity necessary to produce an active antibacterial peptide varied among the different bacteria. The typical Gram-negative rod, *E. coli*, required three B and at least two Ch units present in the peptide for antibacterial activity as exemplified by WRW-OBzl. Additional charge (e.g., RWRW-OBzl) or both bulk and charge (e.g., WRWRW-NH_2) enhanced antibacterial activity. We have recently shown that hexapeptide amides having three tryptophan and three arginine residues represent the most efficient motif for high antibacterial activity against *E. coli*.[10]

The Gram-positive staphylococci, *S. aureus*, MRSA, and MRSE, were generally more susceptible to the peptides in this study. The minimal anti-staphylococcal motif can be defined as the combination of no more than two B and two Ch moieties. An example of such a peptide is RW-OBzl, where the B units are the indole side chain of tryptophan and the benzyl ester group and where the Ch units are the guanidinium group of arginine and the free N-terminal amino group. This dipeptide was surprisingly active compared to many cationic antibacterial peptides of far larger size, such as the protegrin analogue IB-367[17] that is currently in phase III clinical testing for the ability to prevent oral mucositis in cancer patients.[18] An additional anti-staphylococcal motif was the combination of three B moieties and one Ch moiety, which is found in Ind-RW-OBzl. However, peptides with an excess charge and only one B unit such as RWR-NH_2, comprising a one B and three Ch motif, were inactive.

Conclusion. In the present study, we have established that the pharmacophore of cationic antibacterial peptides is surprisingly small, allowing the possibility of designing active peptides far smaller than previously described, in particular regarding anti-staphylococcal activity. These small peptides are particularly effective against multiresistant strains and have no direct lytic effect against human erythrocytes. Admittedly, it is a long journey before these peptides can be transformed into drugs useful for systemic application, but the anti-staphylococcal activity of tri- and dipeptides offers the possibility of oral administration because peptides of this size can be carried across the epithelial membranes by peptide transporters in the GI tract. Furthermore, the low activity against *E. coli* implicates low toxicity against the normal flora of the intestine. The absence of increased activity of D-amino acid containing peptides implies that the peptides are poor substrates for degrading proteases and may therefore show systemic stability. These small peptides thus constitute a novel class of antibacterial peptides addressing several of the existing pharmaceutical concerns with cationic antibacterial peptides.

Experimental Section. Solid-Phase Peptide Synthesis. The peptide amides were prepared on Rink amide support using Fmoc chemistry as described previously.[12]

Solution-Phase Peptide Synthesis. The peptide ester derivatives were prepared in solution using a Boc protecting strategy according to the following general procedure. The unprotected amino acid ester (1 equiv), the Boc-protected amino acid (1.05 equiv), and 1-hydroxybenzotriazole (1-HOBt) (3.6 equiv) were dissolved in DMF (2 mL/mmol amino component) before addition of diisopropylethylamine (DIPEA) (4.8 equiv). The mixture was cooled on ice, and *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU) (1.2 equiv) was added. The reaction mixture was shaken at ambient temperature for 1.5 h. The reaction mixture was diluted by ethyl acetate and washed with citric acid, sodium bicarbonate, and brine.

The solvent was removed under vacuum, and the peptide was deprotected in the dark using 95% TFA.

Preparation of H-Arg-OBzl. A solution of Boc-Arg-OH (2.5 mmol) in 90% methanol (22 mL) was neutralized by addition of aqueous cesium carbonate and evaporated in vacuo to dryness. Residual water was removed by repeated azeotropic distillations using toluene. The dry cesium salt was dissolved in DMF, benzyl bromide (3 mmol) was added, and the solution was stirred for 6 h. The solvent was removed in vacuo, and acetone was added. The resulting suspension was filtered, the filtrate was evaporated, and the product was treated with 95% TFA (4 mL). The title compound was isolated as a hygroscopic solid after trituration with diethyl ether. ^{1}H NMR (D_2O) δ 7.23 (m, 5H), 5.14 (d, 1H, J = 12.7 Hz), 5.00 (d, 1H, J = 12.7 Hz), 4.63 (m, 1H), 2.88 (m, 2H), 1.72 (m, 2H), 1.40 (m, 1H), 1.27 (m, 1H); ^{13}C NMR (D_2O) δ 169.7, 156.8, 134.8, 129.2, 129.1, 128.9, 68.8, 52.6, 40.4, 27.1, 23.9.

Peptide Purification and Analysis. The peptides were purified using reversed-phase HPLC on a Delta-Pak (Waters) C_{18} column (100 Å, 15 μm, 25 mm × 100 mm) with a mixture of water and acetonitrile (both containing 0.1% TFA) as eluent. The peptides were analyzed by RP-HPLC using an analytical Delta-Pak (Waters) C_{18} column (100 Å, 5 μm, 3.9 mm × 150 mm) and positive ion electrospray mass spectrometry (VG Quattro quadrupole mass spectrometer from VG Instruments Inc., Altringham, U.K.). The purity of all peptides was greater than 95%.

Antibacterial Activity. The bacterial strains *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923), methicillin-resistant *S. aureus* (ATCC 33591) (MRSA), and methicillin-resistant *S. epidermidis* (ATCC 27626) (MRSE) were grown in 2% Bacto Peptone water (DIFCO 1807-17-4) until exponential growth was achieved. A standard microdilution technique with an inoculum of 2×10^6 cfu/mL was used. The MIC of the peptides was determined in 1% Bacto Peptone water after incubation of the sample overnight at 37 °C. All peptides were tested in parallel using at least three independent dilutions. The following titer series were employed: 200, 150, 100, 75, 50, 25, 15, 10, 7.5, 5, 2.5, and 1 μg/mL. The susceptibility of the bacterial strains against gentamicin was used as an internal standard during MIC determinations. The results (MIC values in μg/mL) were the following: *E. coli*, 0.12−0.25; *S. aureus*, 0.03−0.06; MRSA, 0.12; MRSE, less than 0.01.

Hemolytic Activity. Freshly isolated human erythrocytes isolated as recently described[19] were incubated for 1 h at 37 °C with 1, 50, 100, 500, and 1000 μg/mL solution of peptide dissolved in PBS. The samples were centrifuged (4000 rpm, 5 min) before the absorbance at 540 nm of the supernatant was measured by a microtiter plate reader. PBS and Triton X100 were used as negative and positive controls, respectively. Peptide concentrations corresponding to 50% hemolysis (EC_{50}) were determined from the dose−response curves.

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