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Monomeric analogues of halocidin

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Halocidin is a heterodimeric antimicrobial peptide isolated from a tunicate, *Halocynthia aurantium.* We used the most active of the two monomers, an 18 residue amidated peptide, as lead structure and determined the role of each amino acid with alanine scanning. The results obtained led to the synthesis of a first generation of analogues with antimicrobial activity. The selectivity towards bacterial *versus* mammalian cells has been explored, as well as the specificity for gram positive (*Staphylococcus aureus* ATCC 25923) *versus* gram negative bacteria (*Escherichia coli* ATCC 25922). GRAVY (grand average of hydropathicity) was used to analyze the results.

Introduction

Bacterial resistance to traditional antibiotics is becoming a dramatic problem. The occurrence of multidrug-resistant pneumococci in communities, and methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococci* in hospital environments has increased greatly, and has culminated in the first documented case of infection caused by vancomycin-resistant *S. aureus* recently reported.[1] The undiscerned use of antibiotics in medicine and agriculture has led to this situation,[2] and attempts to correct it range from a new ruling by the FDA[3] to the search for new antibiotics with new modes of action. New bacterial targets have been defined and validated, as opposed to the classical objectives used by traditional antibiotics.[4] Microbial genomics have revealed a number of these new targets that can potentially lead to new classes of antimicrobial agents.[5,6] Among the new putative targets[7] are antimicrobial peptides and proteins which are part of the innate immunity, a very ancient protection mechanism acting as first line of defence against pathogen aggression of the host.[8–12] Although antimicrobial peptides differ greatly in their sequence and structure, nearly all of them are positively charged. Most are amphiphatic, which reflects their properties to disrupt bacterial membranes and results in their broad spectrum activity towards pathogens.

Very few antimicrobial peptides exist as dimers. The rhesus theta defensin-1 results from the head-to-tail ligation of two truncated α-defensins, thus forming two new peptide bonds. Furthermore, this unusual peptide contains 3 disulfide bridges.[13] Two dimeric peptides have recently been isolated from the tunicate *Halocynthia aurantium*, dicynthaurin and halocidin. These peptides are contained in the haemocytes, phagocytic cells considered to be the functional counterparts of the mammalian leukocytes in protochordates.[14] Dicynthaurin is a homodimer, with each monomer constituted of 30 amino acids.[15] In contrast, halocidin is a heterodimer containing two monomers of 15 and 18 residues. Both amino acid sequences are identical and amidated on the *C*-terminus, with the 15-mer corresponding to an *N*-terminus truncated version of the 18-mer **1**.[16,17] Surprisingly, the 18 residue monomer **1** (WLNAL^{5}LHHGL^{10}NCAKG^{15}VLA-NH_2) and its homodimer form have an antibacterial activity comparable to the natural heterodimer halocidin. On the other hand, the 15 residue monomer and the corresponding dimer showed a weaker antimicrobial activity. Finally, all these peptides were weakly haemolytic, emphasizing their capability to discriminate between bacterial and mammalian membranes and therefore making them suitable lead structures as antimicrobial peptides.

The constant search for new antibiotics prompted us to investigate this intriguing peptide, halocidin. We focused our attention on the more active of the two monomers, the 18 residue peptide **1**. In this paper, we report the Ala-scan carried out on 18-mer peptide **1** with the antibacterial and haemolytic activity of the resulting peptides synthesized: **2** to **19** (Table 1). The results from this Ala-scan indicated structural requirements for optimal activity: a) position 1: an aromatic side chain; b) positions 2, 4, 5, and 17: a bulky side chain; c) positions 7 and 14: a positively charged side chain; position 8: a small aliphatic side chain; e) positions 3 and 11: a hydrophilic side chain. Using these results, a first generation of peptides **20** to **27** has been synthesized and their biological assays reported in Table 2. The results obtained from the Ala-scan were confirmed and for some positions, more details were gained. The hydrophilic side chain in position 11 should be small. On position 8, a small aliphatic side chain was more active on gram positive bacteria whereas a positively charged side chain was more active on gram negative bacteria. A positive charge in position 7, 8, and 11 was beneficial. All the results obtained were analyzed with GRAVY to gain a better understanding of the role of the hydrophobicity in the biological activity of these peptides.

Results and discussion

Ala-scan

Scans have been widely used to obtain insight into the role of each amino acid composing a peptide or a protein. The most frequently used amino acid in scanning is alanine.[18] Alanine, with a methyl group as a side chain, is a small amino acid and does not have much preference for the surface or the inside of a protein. Glycine, with a simple hydrogen atom as a side chain, is a smaller amino acid with no side chain hindrance, consequently glycine residues can adopt unusual dihedral backbone angles whereas alanine lacks any such unusual preferences. Therefore, an alanine residue is neutral from the backbone point of view and it will emphasize the role taken by the side chain of the replaced amino acid.

Halocidin contains three alanine residues in positions 4, 13, and 18 incompatible with alanine scanning (Fig. 1). Not replacing these residues would result in a loss of information about their role. Two possibilities are offered to probe for the role of the β-methyl group of alanine: either choosing a simpler amino acid, glycine, or using a larger amino acid. Glycine is not suitable for flexibility reasons mentioned above, and we therefore chose an amino acid with a bulkier hydrophobic side chain: leucine.

The results provided by the Ala-scan can be divided into three unevenly distributed groups. The first group of peptides (**2**, **3**, **6**, **8**, **10**, **13**, **14**, **15**, and **16**), representing half of the positions scanned (Table 1), displayed higher minimal inhibitory

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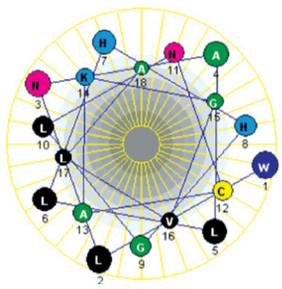

Fig. 1 Helical wheel of peptide **1**.

concentration (MIC) values for both gram positive and negative bacteria, and these peptides were more haemolytic than the reference 18-mer peptide **1**. Replacing Trp 1 with alanine in peptide **2** was unfavourable, highlighting the difference between aromatic and aliphatic side chains. Often, Trp is found at the membrane–aqueous interface,[19] a role that clearly alanine cannot fulfil. As a result, the MIC value for both bacteria was higher. Trp has also been implicated in haemolytic activity,[20] but it is not the case here as the alanine analogue is much more haemolytic. The high haemolytic activity is the result of a higher hydrophobicity as shown by GRAVY:[21] 0.672 for our reference **1** *versus* 0.822 for peptide **2** (Table 1). Replacing Leu 2 and 5 with alanine (peptides **3** and **6**) produced poor results, pointing to the importance of a bulky side chain at these positions. Both analogues **3** and **6** were also more haemolytic. Structural factors are probably responsible for the difference observed in the haemolytic activity of these two peptides as both display the same value for GRAVY. The replacement of His 7 and Lys 14 with alanine gave the expected results, producing haemolytic peptides **8** and **15**, respectively, with poor MIC values. Altering the charged hydrophilic core of antimicrobial peptides generally results in an increased MIC value and haemolytic activity. The value for GRAVY calculated shows that peptides **8** and **15** are indeed much more hydrophobic than our standard **1** (0.950, 0.989, and 0.672 respectively). The net overall positive charges of these peptides are indeed decreased, affecting their self-promoted uptake by bacteria, as well as their capacity to discriminate between negatively charged bacterial membranes and zwitterionic mammalian membranes because of their higher hydrophobicity.[22] An unexpected result was obtained by replacing Gly 9 and 15 with alanine. An easier α-helix formation was expected by exchanging the very flexible Gly with alanine, neutral from the peptide backbone point of view. We anticipated a better MIC but not a less haemolytic peptide as predicted by the value for GRAVY that showed these two peptides **10** and **16**, respectively, to be more hydrophobic than reference **1** (Table 1). Circular dichroism measurements of the reference 18-mer **1** and its analogue **16** with Gly 15 replaced with alanine confirmed our expectation with regard to the increase in α-helicity. Assuming a two state model, the ellipticity at 222 nm is linearly related to the mean helix content f_H of a peptide. This helical content can be calculated using the Lifson–Roig based helix-coil model:[23] $f_H = ([\theta]_{222} - [\theta]_C)/([\theta]_H - [\theta]_C)$, where $[\theta]_{222}$ is the mean residue ellipticity at 222 nm of the calculated spectrum, $[\theta]_C$ is the mean residue ellipticity of the random coil, and $[\theta]_H$ is the mean residue ellipticity of the complete helix. The values of $[\theta]_C$ and $[\theta]_H$ can be determined as follows: $[\theta]_C = 2\,220 - 53T$, and $[\theta]_H = (-44\,000 + 250T)(1 - 3/N_r)$, where T is the temperature in °C, and N_r is the number of residues. The value obtained for the mean helix content f_H is only an assessment that can be overestimated in some cases.[24] We recorded the CD spectra of seven peptides (**1**, **14**, **16**, **19**, **20**, **25**, and **26**) (data not shown). In phosphate buffer, all peptides presented a randomly coiled structure. Upon addition of 50% trifluoroethanol (TFE), a solvent known to promote structural organisation in peptides,[25] all peptides showed the typical features of α-helical structures: two negative minima at 222 and 208 nm and a positive maximum near 195 nm. Our reference peptide **1** showed a helical content of 70%. Simply replacing Gly 15 with alanine increased the helicity of peptide **16** to 81%. Antimicrobial peptides with a too high helical content have been shown to lose their specificity toward bacteria and to become toxic toward mammalian cells.[26] Indeed, the analogue **16** containing alanine in position 15 is more haemolytic than 18-mer **1**. Furthermore, peptide **16** displayed a similar MIC value for gram negative bacteria and a value more than double for gram positive bacteria. Replacing Gly 9 with alanine led to a similar trend, peptide **10** being even more haemolytic. It is reasonable to suspect as the cause for these poor results helicity and hydrophobicity, as predicted from the value calculated for GRAVY. Replacing Cys 12 with alanine also produced poor results. Peptide **13** was more haemolytic than our reference **1** and the MIC values were among the worst obtained despite a GRAVY value calculated showing peptide **13** to be less hydrophobic than our standard **1** (0.633 *versus* 0.672, respectively). The polarizability and polarity of Cys probably account for the results obtained. Finally, the replacement of Ala 13 with a leucine produced peptide **14** displaying MIC values for gram negative bacteria nearly identical to our reference **1** whereas the MIC values for gram positive bacteria were more than twice as high. The haemolytic activity of peptide **14** was much higher than our standard **1** as predicted by the value for GRAVY: 0.783 for peptide **14** *versus* 0.672 for our reference **1**. The lack of discrimination between mammalian and bacterial membranes could then be attributed to the increase in hydrophobicity due to the leucine side chain.

A small second group (peptides **5**, **9**, and **17**) also displayed a worse MIC value but with a similar or reduced haemolytic activity than our reference peptide (Table 1). This group represents only 17% of the scanned positions. Replacing Ala 4 with leucine provided peptide **5** slightly less active against gram positive and negative bacteria but retaining the same selectivity for gram positive bacteria as our reference **1**. Furthermore, peptide **5** was more specific towards bacterial membranes as illustrated by its haemolytic activity which was lower than our standard **1** despite a higher value for GRAVY (0.783 *versus* 0.672). This result is in sharp contrast with the much higher haemolytic activity obtained when replacing Ala 13 with leucine in peptide **14**. Replacing His 8 with alanine gave unexpected results. Peptide **9** was nearly as active on gram positive bacteria as our reference peptide **1**. It also showed a weaker activity on gram negative bacteria. This resulted in a better selectivity for gram positive bacteria when compared to our reference **1**. Peptide **9** was not haemolytic at the highest concentration studied and represents the lowest haemolytic activity obtained from the alanine scanning although the value for GRAVY for peptide **9** (0.950) was much higher than our standard **1** (0.672). Replacing Val 16 with alanine produced peptide **17** which was less haemolytic, as predicted by the value for GRAVY (0.539 *versus* 0.672), but also less active on both gram positive and negative bacteria.

The third group (peptides **4**, **7**, **11**, **12**, **18**, and **19**), representing 33% of the positions investigated, is more heterogeneous (Table 1). Although these peptides are more haemolytic than (or in one case as haemolytic as) the reference 18-mer **1**, the MIC values obtained are similar or better either for gram positive and/or gram negative bacteria. Replacing Asn 3 with alanine produced peptide **4** with a slightly higher MIC value for gram positive bacteria, but a lower MIC value for gram negative bacteria. Alanine abolished the selectivity between gram positive and negative bacteria, pointing to the importance of a hydrophilic amino acid in position 3. However peptide **4** was much more haemolytic than our standard **1**. The CD spectrum recorded for peptide **4** showed a α-helical configuration upon addition of 50% TFE. The dramatic rise in haemolytic activity can hardly be explained by the slight increase in helicity when compared to our reference, from 70% for **1** to 73% for **4**, whereas the increase in hydrophobicity, as shown by the value for GRAVY (0.967 *versus* 0.672), is more likely implicated. The

Table 1 Ala-scan

peptide	modification	*S. aureus*[a]	*E. coli*[a]	AAA[b]	Haemolysis[c]	MW calc.	MW obs.	HPLC[d]	GRAVY[e]
1	18mer[f]	14.7	29.4	0.92	5.5%	1929.34	1929.5	15.6	0.672
2	$W^{1} \rightarrow A$	>71.7	>71.7	1.12	37.6%	1814.20	1815.0	14.8	0.822
3	$L^{2} \rightarrow A$	19.4	38.7	1.21	14.4%	1887.26	1887.9	14.6	0.561
4	$N^{3} \rightarrow A$	18.1	18.1	1.13	41.6%	1886.31	1887.0	16.2	0.967
5	$A^{4} \rightarrow L$	22.6	45.1	1.41	1.4%	1971.42	1972.7	15.9	0.783
6	$L^{5} \rightarrow A$	17.9	71.7	1.12	61.7%	1887.26	1887.6	14.6	0.561
7	$L^{6} \rightarrow A$	14.9	59.5	0.93	55.7%	1887.26	1887.9	14.5	0.561
8	$H^{7} \rightarrow A$	38.7	77.4	1.21	54.9%	1863.28	1863.6	16.7	0.950
9	$H^{8} \rightarrow A$	15.5	62.1	0.97	0%	1863.28	1864.0	17.2	0.950
10	$G^{9} \rightarrow A$	37.1	37.1	1.16	36.1%	1943.36	1943.7	17.1	0.794
11	$L^{10} \rightarrow A$	14.1	56.3	0.88	62.2%	1887.26	1888.3	14.7	0.561
12	$N^{11} \rightarrow A$	14.9	14.9	0.93	40.6%	1886.31	1886.0	16.1	0.967
13	$C^{12} \rightarrow A$	>71.0	>71.0	1.11	15.2%	1897.27	1897.2	15.7	0.633
14	$A^{13} \rightarrow L$	33.0	33.0	1.03	46.5%	1971.42	1972.7	17.2	0.783
15	$K^{14} \rightarrow A$	72.3	>72.3	1.13	42.4%	1872.24	1872.8	16.3	0.989
16	$G^{15} \rightarrow A$	33.3	33.3	1.04	32.0%	1943.36	1943.7	16.4	0.794
17	$V^{16} \rightarrow A$	38.1	76.2	1.19	4.0%	1901.28	1901.5	15.1	0.539
18	$L^{17} \rightarrow A$	11.8	>47.4	0.74	6.1%	1887.26	1887.1	14.8	0.561
19	$A^{18} \rightarrow L$	18.7	9.4	1.17	26.0%	1971.42	1972.7	16.2	0.783

[a] Minimal Inhibitory Concentration in μg ml^{-1}. [b] Amino Acid Analysis (mg ml^{-1}). [c] Haemolysis in % for a concentration of 230 μg peptide ml^{-1}. [d] Analytical HPLC, retention time in min. [e] Grand average of hydropathicity. [f] $WLNAL^{5}LHHGL^{10}NCAKG^{15}VLA\text{-}NH_2$.

same results were obtained when Asn 11 was replaced with alanine in peptide **12**, albeit with slightly better MIC values. It appears therefore that a low haemolytic activity is achieved when a hydrophilic amino acid is present in positions 3 and 11. Replacing Asn with a more hydrophobic amino acid results in a loss of selectivity between mammalian and bacterial membranes. When Leu 6 was substituted with alanine in peptide **7**, the MIC value displayed for gram positive bacteria was similar to our reference **1**, whereas the MIC value for gram negative bacteria was doubled. Thus, a small lipophilic side chain in position 6 leads to a higher specificity for gram positive bacteria despite a lower value for GRAVY (0.561 *versus* 0.672). However, the replacement of Leu 6 with alanine led to peptide **7** being much more haemolytic than our standard **1**. Substituting Leu 10 with alanine in peptide **11** produced similar results for the MIC values and yielded the most haemolytic peptide of the Ala-scan. In the same manner, exchanging Leu 17 with alanine generated peptide **18** which was more specific for gram positive bacteria. Peptide **18** is the only peptide belonging to this group that displays a haemolytic activity similar to our 18-mer reference **1** in agreement with the value calculated for GRAVY (0.561 *versus* 0.672). It is to be noted that all three leucine residues, 6, 10, and 17, are contiguous on the helical wheel (Fig. 1).[27] Peptide **19** displayed a haemolytic activity higher than our standard **1**. This lack of selectivity between mammalian and bacterial membranes can be attributed to the increased lipophilicity of leucine, as shown by the value for GRAVY (0.783 *versus* 0.672), combined with a slightly higher helicity of this peptide: 77% for **19** *versus* 70% for our standard **1**.

Overall, the Ala-scan gave several indications about the structural requirements for optimal activity: a) an aromatic side chain in position 1; b) a bulky side chain in positions 2, 4, 5, and 17; c) a positive charge in positions 7 and 14; d) a small aliphatic side chain in position 8; e) a hydrophilic side chain in positions 3 and 11. Finally, the other amino acids should be conserved as their replacement did not yield any substantial gain in activity.

Analogues

Using the results from the Ala-scan, we synthesized 8 new peptides (**20** to **27**) in which one or several (**25**) amino acids were replaced (Table 2). The data obtained can be distributed among four groups of two peptides each. The first group includes peptides **20** and **21** displaying a worse MIC and that are as or more haemolytic than our reference peptide **1**. Replacing Gly 9 with leucine yielded peptide **20** with a MIC value higher for gram positive and gram negative bacteria. As expected, these higher values are twice the corresponding MIC values noted for the replacement of Gly 9 with alanine in peptide **10**. Although the antimicrobial activity follows a logical stepwise escalation pattern with Gly 9 > Ala 9 > Leu 9, it is not the case for the haemolytic properties of these 3 peptides **1**, **10**, and **20**. Whereas replacing Gly 9 with alanine produced peptide **10** which is much more haemolytic, the substitution of Gly 9 with leucine yielded peptide **20** which is only slightly more haemolytic than our reference **1**. It is surprising that using a more hydrophobic amino acid, as shown by the value calculated for GRAVY (Table 2), and restricting the rotational freedom as revealed with the increase in helicity (79% for **20** *versus* 70% for **1**) does not have a more pronounced effect on the haemolytic activity of this peptide. It appears that antimicrobial but not haemolytic activity is governed by hydrophobic properties at position 9 of peptide **1**. Replacing Asn 3 with leucine in peptide **21**, the second peptide belonging to this group, confirmed in part our hypothesis for the requirement of a hydrophilic amino acid in position 3 for a selective antimicrobial activity favouring gram positive bacteria. Indeed, leucine in position 3 in peptide **21** did not show any apparent selectivity for either group of pathogens tested. Furthermore, the trend for the MIC value of gram positive bacteria was confirmed: a slightly higher value with alanine and nearly 6 times higher with leucine. The pattern for the MIC value of gram negative bacteria was reversed: if alanine displayed a MIC value nearly twice as low, leucine showed a corresponding value much higher. The haemolytic activity, 8 times that of our reference peptide **1**, was in the same range for both replacements with either alanine (**4**) or leucine (**21**) and in agreement with the value calculated for GRAVY (Table 2).

In the second group, peptides **23** and **27** had worse MIC values but were less haemolytic than our reference 18-mer **1**. Replacing His 8 with lysine produced peptide **23** with no selectivity toward either bacteria, whereas alanine in position 8 in peptide **9** was more selective for gram positive bacteria. Compared to peptide **1**, the MIC value of peptide **23** for gram positive bacteria was higher whereas it was only slightly higher for gram negative bacteria. It appears that in position 8, a small hydrophobic side chain is not favourable against gram negative bacteria whereas a polar hydrophilic side chain at the same position disfavours gram positive bacteria. Replacing His 8 with lysine gave peptide **23**, which was slightly less haemolytic than our reference **1**, in agreement with the value calculated for GRAVY (0.633 *versus* 0.672), whereas peptide **9** with alanine in position 8 was surprisingly not haemolytic despite a higher value for GRAVY (Table 2). Since halocidin exists as a heterodimer containing a

Table 2 First genaration analogues

peptide	modification	*S. aureus*[a]	*E. coli*[a]	AAA[b]	Haemolysis[c]	MW calc.	MW obs.	HPLC[d]	GRAVY[e]
1	18mer[f]	14.7	29.4	0.92	5.5%	1929.34	1929.5	15.6	0.672
20	$G^{9} \rightarrow L$	75.5	>75.5	1.18	6.6%	1985.45	1987.1	19.0	0.906
21	$N^{3} \rightarrow L$	>81.3	>81.3	1.27	43.5%	1928.39	1930.2	17.1	1.078
22	$H^{7} \rightarrow K$	10.1	10.1	1.26	43.6%	1920.37	1919.0	16.0	0.633
23	$H^{8} \rightarrow K$	36.8	36.8	1.15	3.8%	1920.37	1920.1	15.8	0.633
24	$N^{11} \rightarrow K$	19.4	19.4	1.21	39.8%	1943.41	1943.3	15.3	0.650
25	$H^{7,8} + N^{11} \rightarrow K$	32.0	16	1.00	2.2%	1925.47	1924.2	15.9	0.572
26	$W^{1} \rightarrow$2-Nal	10.4	10.4	1.30	0%	1940.36	1939.5	15.6	ND
27	$C^{12} \rightarrow C^{Acm}$	>92.2	>92.2	1.44	0%	2000.42	1999.7	16.2	ND

[a] Minimal Inhibitory Concentration in μg ml^{-1}. [b] Amino Acid Analysis (mg ml^{-1}). [c] Haemolysis in % for a concentration of 230 μg peptide ml^{-1}. [d] Analytical HPLC, retention time in min. [e] Grand average of hydropathicity. [f] WLNAL^{5}LHHGL^{10}NCAKG^{15}VLA-NH_2.

disulfide bridge, we anticipated that substitution on the cysteine side chain should not be detrimental. We therefore replaced Cys 12 with *S*-acetamidomethyl cysteine (Cys^{Acm}). This produced peptide **27** seemingly devoid of selectivity toward either gram positive or gram negative bacteria and with unexpected properties. Replacing Cys 12 with either alanine (**13**) or Cys^{Acm} (**27**) showed the same trend: both peptides gave higher MIC values for gram positive and gram negative bacteria, these values being more pronounced for Cys^{Acm}. Peptide **27** with Cys^{Acm} in position 12 was not haemolytic at the highest concentration tested, whereas the corresponding alanine analogue **13** was more haemolytic than our reference peptide **1**. This behaviour has tentatively been attributed to the hydrophilic character of the acetamidomethyl group.

The third group contained two haemolytic peptides, **22** and **24**, with nearly identical or lower MIC values than our reference peptide **1**. Although replacing His 7 with lysine in peptide **22** did not show any selectivity for a particular pathogenic group, the MIC values observed for gram positive and negative bacteria were lower, whereas the corresponding substitution with alanine (**8**) displayed higher MIC values, albeit with the same selectivity as the parent peptide **1**. Therefore a polar hydrophilic side chain in position 7 is able to induce better MIC values for gram positive and gram negative bacteria. This advantage has to be tempered with the haemolytic property of this peptide which is much higher than our reference peptide **1** despite a lower value for GRAVY (Table 2), whereas alanine in position 7 (**8**) is even more haemolytic. Striking differences were noted when comparing the results obtained upon replacing His 7 and 8 with lysine in peptides **22** and **23**. The only similarity between both peptides was the lack of specificity toward gram positive or negative bacteria. Whereas lysine in position 7 (**22**) resulted in lower MIC values for both pathogens, it induced higher MIC values in position 8 (**23**). The reverse trend occurred for the haemolytic activity: it was increased with lysine in position 7 (**22**) but decreased with the same amino acid in position 8 (**23**). These dissimilarities could be explained by the fact that His 7 and 8 lie at a 45 degree angle to each other on the helical wheel (Fig. 1). Furthermore, their surrounding is quite different, a fact that the value calculated for GRAVY failed to convey (Table 2). His 7 is positioned in a rather hydrophilic environment, its neighbours being on one side Lys 14 and Asn 3 and on the other Ala 18 and Asn 11. In contrast, the setting of His 8 is somewhat more hydrophobic, being surrounded by Gly 15 and Ala 4, and Trp 1 and Cys 12. Substituting Asn 11 with lysine gave the second peptide (**24**) of this group which is not selective. Whereas the MIC value for gram positive bacteria is slightly higher than the parent peptide **1**, the MIC value for gram negative bacteria is lower. The slight gain in antimicrobial activity is offset by a haemolytic activity much higher than our reference peptide **1** despite a lower value for GRAVY (Table 2). Overall, replacing Asn 11 with alanine (**12**) produced better MIC values than with lysine (**24**), but both substitutions led to a higher haemolytic activity when compared to peptide **1**. At position 11, a side chain combining small size with a polar hydrophilic character seems to be required for having concurrently low MIC values and a low haemolytic activity. In this regard, serine is a promising candidate and will be used in the next generation of modified analogues.

The fourth and last group encompasses the most interesting peptides, **25** and **26**. For the first peptide **25**, we chose to increase the overall net positive charge and replaced His 7, His 8, and Asn 11 with lysine. To our delight, we found that the selectivity of peptide **25** for gram positive and gram negative bacteria was the reverse of the parent peptide **1**. This new peptide **25** was indeed twice as selective for gram negative bacteria. This reverse selectivity was achieved through a higher MIC value for gram positive bacteria and an MIC value nearly twice as low for gram negative bacteria. Peptide **25** was also less haemolytic than our reference peptide **1**. Therefore an increase and clustering of the positive charges resulting for this peptide in a lower value for GRAVY (0.572 *versus* 0.672) combined with a preserved helicity (68% for this peptide *versus* 70% for the parent peptide) were revealed to be beneficial. In the last synthesized peptide **26**, Trp 1 was replaced with a non-natural amino acid: 3-(2-naphthyl)-alanine. Although both amino acids are aromatic, they present however different characteristics. Tryptophan possesses a hydrogen bonding capability whereas 3-(2-naphthyl)-alanine is more lipophilic, less polar, and with a larger surface area than tryptophan. Both residues on the side chains are positioned asymmetrically, but the volume of space covered by rotation of the tryptophan side chain is much larger because of a more pronounced asymmetry. As a consequence, the potential for steric clashes is higher for tryptophan. This asymmetrical orientation also projects the naphthyl ring of 3-(2-naphthyl)-alanine further away from the peptide backbone than for the indole ring of tryptophan.[28] Therefore 3-(2-naphthyl)-alanine has a higher capacity to bury itself in a lipophilic environment, whereas tryptophan is known to be able to locate itself preferentially at the membrane–aqueous interface, particularly in the region near the lipid carbonyls.[29] Replacing Trp 1 with 3-(2-naphthyl)-alanine in peptide **26** did not provide any selectivity toward gram positive or gram negative bacteria, in contrast to the parent peptide **1**. On the other hand, the MIC values obtained for **26** were lower for gram positive and gram negative bacteria. To our satisfaction, peptide **26** was not haemolytic at the highest concentration studied. These results are in contrast with the data obtained when Trp 1 was replaced with alanine (**2**), confirming the importance of an aromatic side chain over an aliphatic one at position 1 of the peptide.

Overall, the results obtained from these analogues confirmed the results from the Ala-scan with some additional information. Glycine and cysteine should be conserved in position 9 and 12, respectively, despite a better haemolytic activity with a hydrophilic side chain in position 12. A hydrophilic amino acid is preferred in positions 3 and 11. The side chain should be small in position 11, suggesting serine as a promising candidate. More details were obtained for position 8: a small aliphatic side chain was more active on gram positive bacteria whereas a positively charged side chain was more active on gram negative bacteria. A positive charge in positions 7, 8, and 11 was beneficial.

Although lysine in position 7 gave good MIC values, the haemolytic activity increased. This is probably correlated with the hydrophobic character of the methylene groups of the side chain. A hydrophilic amino acid is preferred to lysine in position 11. Finally, an aromatic side chain is necessary in position 1.

Conclusion

The theoretical values obtained from calculating the GRAVY coefficient were coherent with the HPLC retention time of the peptides synthesized for the Ala-scan with 3 exceptions (**2**, **10**, and **13**) over the 18 analogues. Only 2 of the 6 values were consistent for the first generation analogues, with 2 peptides not included as only the proteogenic amino acids are considered for the GRAVY coefficient. A possible explanation for these discrepancies would be that the GRAVY coefficient is calculated for peptides with a carboxylic acid at the *C*-terminus. All the synthesized peptides are amidated on their *C*-terminus, and *C*-terminus amidation is known to alter the dipole moment of α-helical peptides.[30] Furthermore, the GRAVY coefficient did not display the subtleties of values shown by the HPLC retention time. The GRAVY coefficient for the replacement of Leu 2, 5, 6, 10, and 17 is the same whereas the retention time value discriminates between each peptide, and therefore their physical properties.

Using alanine scanning we investigated one of the two peptides constituting the heterodimer halocidin. The percentage of successful hits, even considering the lax criteria of "as good as" and "better than", was fairly low (25%). These results were very scattered, with only 2 substitutions (Asn 11, and Leu 17) displaying two combined improvements. However, we gained several indications about the structural requirements for optimal activity. They are summarised as follow: a) an aromatic side chain in position 1; b) a bulky side chain in positions 2, 4, 5, and 17; c) a positive charge in positions 7 and 14; d) a small aliphatic side chain in position 8; e) a hydrophilic side chain in positions 3 and 11. Finally, the other amino acids should be conserved as their replacement did not yield any substantial gain in activity. These findings allowed us to synthesize a first generation of monomeric analogues with a better success rate. Using the more stringent parameter "better than", the percentage of successful hits rose to 42%. The results obtained from the Ala-scan were confirmed by these analogues and for some positions, more details were gained. The hydrophilic side chain in position 11 should be small. On position 8, a small aliphatic side chain was more active on gram positive bacteria whereas a positively charged side chain was more active on gram negative bacteria. A positive charge in positions 7, 8, and 11 was beneficial. These results culminated in peptide **26** with an overall improvement for both strains of bacteria and for the haemolytic activity due to the incorporation of a non-natural amino acid in position 1: 3-(2-naphthyl)-alanine.

We were also able to identify specific positions with an impact on the MIC value of gram positive and/or gram negative bacteria, with or without concomitant influence on the haemolytic activity. The prediction of the additivity of these properties being impossible, we are currently investigating the homo or heterodimerisation of such peptides.

Experimental

Peptide synthesis

All peptides were manually synthesized by standard solid phase peptide synthetic methods using Fmoc chemistry.[31] All L-amino acids were protected with an Fmoc group on the *N*-terminus (Novabiochem and Aldrich-Fluka). The following protective groups were used on the amino acid side chains: Boc for Trp and Lys, Pbf for Arg, Trt for His and Cys, and Acm for Cys. The resin used was Tentagel S RAM (50 mg, loading 0.24 mmol g^{-1}, Fluka), and the following coupling conditions were used: Fmoc-AA-OH/TBTU/HOBt/DIPEA (1 : 1 : 1 : 1.5) in NMP with double coupling (2 h + 30 min). Cleavage of the peptides from the resin was achieved using 0.5 ml of TFA/TIS/H_2O (95 : 2.5 : 2.5) for 2 h, yielding the corresponding peptides amidated on the *C*-terminus. The peptides were then precipitated and washed in cold diethyl ether, and centrifuged. The resulting white pellets were dissolved in 10% acetic acid–water and lyophilized. The peptides were identified using LC-MS and then purified by preparative RP-HPLC. The purified peptides were finally characterized by MALDI-TOF mass spectrometry and their purity confirmed with analytical RP-HPLC.

RP-HPLC

The purity of the peptides was assessed with analytical RP-HPLC (Waters C_{18} column, Delta-Pak 100 Å 15 μm, Millipore) using a Waters 600E system equipped with Millennium software. Samples were chromatographed at a flow-rate of 1.5 ml min^{-1}, starting with 0.1% aqueous TFA (buffer A) for 10 min and increasing over 45 min to 0.1% TFA in CH_3CN/H_2O (9 : 1) (buffer B), and the detection was performed at 220 nm.

Purification of the peptides was achieved by preparative RP-HPLC (Waters C_{18} column, SymmetryPrep™, 7 μm, Millipore). Samples were initially chromatographed at a flow-rate of 1 ml min^{-1} starting with buffer A/buffer B (9 : 1) for 10 min and increasing over 60 min to buffer A/buffer B (4 : 6) and finally to buffer B over an additional 8 min (flow-rate: 4 ml min^{-1}). The detection was performed at 220 nm.

LC-MS

Identification of the peptides was accomplished using LC-MS (Bruker Esquire Mass Spectrometer). The LC part consisted of a HP 1100 equipped with a Vydac C18 column (cat #238MS215) and a diode array detector. Samples were chromatographed at a flow-rate of 0.25 ml min^{-1} starting with 0.1% aqueous TFA (buffer A) and increasing over 25 min to 0.1% TFA in CH_3CN/H_2O (4 : 1) (buffer B), finally increasing to buffer B over 10 min. The detection was performed at 220 nm.

MALDI-TOF mass spectrometry

Characterization of the purified peptides was completed by MALDI-TOF mass spectrometry (VG Tof Spec E Fisons spectrometer) using α-cyano-*p*-hydroxycinnamic acid (Aldrich, Steinheim, Germany) as matrix. Substance P and ACTH (Sigma, St. Louis, MO, USA) were used as calibrants.

Amino acid analysis

On a stock solution of peptide (about 1 mg ml^{-1}), amino acid analysis was performed (Waters PicoTag[54] analyzer) after hydrolysis of the samples with 6 N aqueous HCl 0.1% at 110 °C. The concentration of each solution was then determined by including a standard, α-aminobutanoic acid. This stock solution was then used in the antimicrobial and haemolytic assays as well as for the circular dichroism spectroscopy, and the results from amino acid analysis were used to correct the different values obtained.

Minimal inhibitory concentration and bacterial strains

The method used to determine the minimal inhibitory concentration (MIC) of the peptides is based on the modified[32] version of the broth micro-dilution assay by the National Committee of Laboratory Safety and Standards.[33] The purified peptides were dissolved in 1% DMSO (Aldrich) resulting in a stock solution with a peptide concentration of 1 mg ml^{-1}. The peptide solutions were then sterile filtered using Whatman Mini-Uniprep™ syringeless filters (0.45 μm PP polypropylene, Sigma). The stock peptide solution was further diluted to give a final concentration of 0.64 μg $μl^{-1}$ of peptide, 0.2% bovine serum albumin (Sigma) and 0.01% acetic acid. Ampicillin (Sigma) was used as an internal standard against *Escherichia coli* ATCC 25922

and *Staphylococcus aureus* ATCC 25923 with 0.25 mg ml^{-1} and 0.01 mg ml^{-1} ampicillin, respectively.

Briefly, serial twofold dilutions of the peptide were made in 0.2% BSA and 0.01% acetic acid solution in sterile 96-well U-bottom polypropylene microtiter plates (Costar; Corning Incorporated, Corning, N.Y.) and gave test concentrations ranging from 64 μg to 2 μg peptide ml^{-1}. Each well was inoculated with 100 μl of *E. coli* or *S. aureus* in supplemented Mueller–Hinton broth (Oxoid, Hampshire, England) to a final concentration of approximately 2×10^5 cfu ml^{-1}. The MIC value was taken as the lowest peptide concentration at which visible bacterial growth was inhibited after 24 h of incubation at 37 °C. Viable bacteria were detected by adding 30 μl of a 3 mmol MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Thiazolyl blue, Sigma)[34] solution to each well and incubating the plates for a further 30 min. All MIC determinations were the results of 3 independent experiments performed in duplicate and the value was corrected using the amino acid analysis results.

Haemolysis assay

Fresh human blood (Blood bank, Rigshospitalet, Copenhagen, Denmark) was centrifuged at 2000 g and the pellet was washed 3 times with cold phosphate buffer saline (PBS) 10 mM pH 7.4. The erythrocytes were brought to a final concentration of ≈1% by dilution with cold PBS. Briefly, serial twofold dilutions were made from 100 μl of peptide stock solution and 100 μl of PBS in sterile 96-well U-bottom polypropylene microtiter plates (Costar; Corning Incorporated, Corning, N.Y.) and gave test concentrations ranging from 250 μg to 1.9 μg of peptide ml^{-1}. In each well, 100 μl of 1% erythrocytes suspension was added and mixed. The plates as well as a negative haemolysis control (100 μl of 1% erythrocytes suspension and 100 μl of PBS) and a positive haemolysis control (100 μl of 1% erythrocytes suspension and 100 μl of 1% Triton X-100) were incubated for 1 h at 37 °C. The plates were then centrifuged at 1500 g. An aliquot of supernatant (100 μl) from each well was then pipetted into a new polystyrene microtiter plate, and the absorbance at 414 nm was read on an ELISA-reader. The percentage of haemolysis was determined as (A_{414} peptide − A_{414} blank)/(A_{414} 1% Triton X-100 − A_{414} blank) × 100, and was corrected with the results from the amino acid analysis.[35]

Circular dichroism

CD spectra were recorded using a Jasco model 710 spectropolarimeter. The optical rotation was calibrated using *d*-camphorsulfonic acid. Two scans were recorded between 280 and 195 nm using a rectangular quartz cell of 0.1 cm pathlength at 25 °C and averaged. The background spectra were recorded in the same solvent without peptide and then subtracted. Peptide stock solution were at an approximate concentration of 100 μg ml^{-1} in either 10 mM phosphate buffer pH 7.0 or 10 mM phosphate buffer pH 7.0 containing 50% TFE. Correction with the results from amino acid analysis gave the final exact concentration. Data were fitted following Juban *et al.*[36]

GRAVY

The grand average of hydropathicity (GRAVY) of the corresponding carboxylic acid peptides was calculated with ProtParam program.[21]

The window size used was $n = 7$, with a relative weight at the edges of the window compared to the centre of 10%. The weight variation model was exponential, and the Kyte–Doolittle hydropathicity scale was employed.[37] A large value is indicative of a hydrophobic peptide. The value of GRAVY for two peptides, **26** and **27**, containing nonnatural amino acid (*S*-acetamidomethyl cysteine and 3-(2-naphthyl)-alanine) was not determined.

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