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Synthetic magainin analogues with improved antimicrobial activity

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Based on modifications to enhance the α-helical structure of the broad spectrum antibiotic magainin 2, a series of analogues have been synthesized which display an increase up to two orders of magnitude in antimicrobial activity and, in the most favorable case, no appreciable increase in hemolytic activity over magainin 1 at the concentrations tested.

Peptide design; Amphiphilic helix; Antibiotic activity; Structure-function relationship

1. INTRODUCTION

The observation that the African clawed frog *Xenopus laevis*, widely used for laboratory work, was remarkably free of infection in water filled with microbes after incisions through both skin and muscular layers of the abdomen into the peritoneum prompted the isolation of the active principles which permit infection-free wound healing in this animal [1]. These principles consist of two peptides (denoted magainin 1 and 2) of 23 amino acid residues differing at positions 10 and 22. They inhibit growth of Gram-positive and Gram-negative bacteria and fungi and induce osmotic lysis of protozoa [1,2]. Magainin peptides were found to be identical with those which had earlier been isolated, characterized and named PGS peptides by Giovannini et al. [3].

Calculation of the potential for α-helix formation by the Chou-Fasman principles [4] and projection of the helix by the Schiffer-Edmundson method [5] revealed that the peptides might have amphiphilic α-helical structures that are important for their antimicrobial activity. Other polypeptides with amphiphilic helical structures have been shown to interact with cell or organelle membranes [6-8]. We report here synthesis of analogues with modifications designed to enhance the amphiphilic α-helical structures and demonstrate that they increase growth inhibitory activity toward a wide variety of microbes by between one and two orders of magnitude.

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2. EXPERIMENTAL

2.1. *Peptide synthesis and purification*

Magainin 1 and analogues of magainin 2 designated A, B, C, D, E, F, G and H whose sequences are shown in table 1 were synthesized by the standard Merrifield solid-phase method with benzhydrylamine resin and coupling procedures using symmetric anhydrides or active esters of *t*-butyloxycarbonyl amino acids as described previously [9]. Anhydrous HF cleavage of the peptides from the resin after completion of the synthesis was performed according to the previously described procedure [9]. Purification of the peptides was accomplished by Sephadex G-25 gel chromatography followed by reverse-phase HPLC on a Vydac C4, 300 A, 5 μm packing column with various eluting gradients composed of acetonitrile and 0.01 M trifluoroacetic acid. In all cases, amino acid composition revealed theoretical recoveries of all amino acids within experimental error and HPLC peak integration at 215 nm indicated >93% purity of the products. Magainin 2 was synthesized by Applied Biosystems, Inc. (Foster City, California) and was purified as above.

2.2. *Circular dichroism*

CD spectra were measured in either 50 mM potassium phos-

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phate buffer, pH 7.0, or 40% (vol/vol) trifluoroethanol (Sigma) in 50 mM potassium phosphate buffer, pH 7.0, on a Jasco model J-5000A spectropolarimeter with a cell path length of 5 mm. Two scans per sample were performed over the wavelength range 250–200 nm. A statistical computer program developed by Provencher [10] was employed for the calculation of α-helical and β-sheet contents.

2.3. *Antimicrobial assay*

The antimicrobial activities of magainin peptides and analogues were assayed by procedures based on the macrodilution method of Jones et al. [11] with modifications. Briefly, different concentrations of peptides were added to 2 ml of trypticase soy broth (BBL) containing the inocula of the test organisms adjusted to 10^5 and 10^6 CFU/ml. Microbial growth was determined by the increase in OD_{600}, after incubation of the tubes at 35°C for 6–9 h depending on the growth rate. The lowest concentration that resulted in complete inhibition of growth was recorded as the 100% minimal inhibitory concentration. The 50% minimal inhibitory concentration was determined from the plots of growth vs concentration of peptide.

2.4. *Erythrocyte hemolysis assay*

To 75 × 12 mm borosilicate test tubes containing a predetermined amount of dried peptide in duplicate, 2.5 ml of diluted human erythrocyte suspension (10%, v/v) in isotonic phosphate-buffered saline was added. After gentle mixing and incubation for 10 min at 37°C, tubes were centrifuged at 3000 × g for 10 min. The supernatant was separated from cells and debris, diluted if necessary, and OD_{350} measured. 100% hemolysis was obtained by using 0.1% Triton X-100 [1].

3. RESULTS

3.1. *Magainin analogues*

From the examination of the primary sequences of magainin peptides elucidated in this laboratory [2] and that of Giovannini et al. [3], we have calculated by the Chou-Fasman principles [4] that magainins have a high potential for α-helix formation. Projection of the helix by the Schiffer-Edmundson method revealed it to be amphiphilic. In order to enhance the helix formation, we modified low helical propensity residues of the sequence at Gly^{13}, Gly^{18} and Ser^{8} of the magainin 2 peptide by substitution of Ala. As expected, the helical wheel diagram (fig.1) shows that the modified peptides maintain an amphiphilic profile very similar to that of the magainins. The magainin 2 sequence was chosen because of its natural abundance and its higher antimicrobial activity than magainin 1 [2].

Modifications of the two termini by amidation of Ser^{23} and acylation of Gly^{1} with acetyl or β-alanyl were expected to stabilize the helical conformation and to lower the susceptibility to exopeptidase action. A D-Ala modification intended to disrupt stretches of helical structure, and thus to demonstrate the importance of that structure for the antimicrobial activity, was also performed. Table 1 shows amino acid sequences of magainin 1, 2 and eight analogues designated A, B, C, D, E, F, G and H.

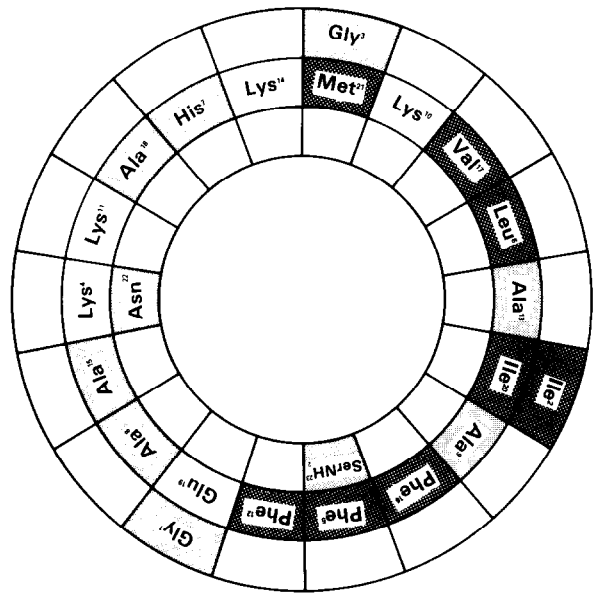

Fig.1. α-Helical wheel diagram of magainin analogue B. The depth of shading corresponds with the hydrophilicity values assigned by Hopp and Woods [12].

3.2. *Circular dichroic measurement*

CD of magainin 1, and 2, and four analogues, B, C, F and G, were measured in aqueous phosphate buffer and 40% trifluoroethanol between the range 200 and 240 nm. The helical contents calculated from the CD spectra by the method of Provencher [10] are summarized in table 2. It is apparent that both natural and modified peptides displayed no α-helix in the aqueous buffer. In the presence of 40% trifluoroethanol, all these peptides displayed an α-helical conformation indicating that a conformational change had occurred in a hydrophobic environment. There is no concentration dependence for the spectra of analogues detected after two-fold dilution. The α-helical contents of the two natural peptides are 24 and 26%, respectively, whereas the helical contents of the modified peptides are more

Table 1

Peptide sequence of magainins and analogues

Peptide	Sequence
	1 5 10 15 20 23
Magainin 2	G-I-G-K-F-L-H-S-A-K-K-F-G-K-A-F-V-G-E-I-M-N-S
Magainin 1	----------G---------- K--
A	βA----------A---------A---------A----------NH_2
B	----------A---------A---------A----------NH_2
C	*N*-Acetyl----------A---------A---------A----------NH_2
D	βA----------A---------A---------A----------NH_2
E	βA----------A---------A----------NH_2
F	----------A---------A----------NH_2
G	βA----------A---------A----------NH_2
H	----------a-------a---------a----------NH_2

All sequence modifications indicated are based on magainin 2. One letter amino acid abbreviation is used. βA, β-alanine; a, D-alanine

than twice higher than those of the two natural peptides. The acetylation of Gly^1 did not appear to enhance the helical conformation.

3.3. *Antimicrobial and hemolytic activities*

The antimicrobial activities of magainin peptides and analogues were assayed against Gram-positive and Gram-negative bacteria as described in section 2. The determination of 100% inhibitory concentration appears to be more consistent among assays than that of 50% inhibitory concentration. The data of 50% inhibitory concentration are listed for the purpose of allowing further comparison of low potency magainin peptides and high potency analogues. As shown in table 3, analogues A, B, D, E, F, and G increased in antimicrobial activity between one and two orders of magnitude over magainin 1 or 2. They clearly have the same spectrum of activity as the naturally occurring peptides. The H analogue which contains D-Ala instead of L-Ala at three modification sites yielded no appreciable activity in all microorganisms tested indicating the conformational requirement for antimicrobial activity.

Human erythrocyte hemolytic activity was measured for magainin 1 and analogues A, B, C, F, G and H in comparison with the hemolytic principle, melittin, from honey bee venom. The result is illustrated in fig.2. Neither magainin 1 nor analogue H showed hemolytic activity at 250 μg/ml of heparinized blood. At a peptide concentration of 500 μg/ml (not shown), analogue H did not cause hemolysis whereas magainin 1 showed only 3% hemolysis. However, the other analogues exhibited appreciable hemolysis at 100 μg/ml which was approximately equivalent to 1/100 the potency of melittin. Analogue G showed less than 1% hemolytic activity at 200 μg/ml. These results indicate that the hemolytic activity of the analogues generally increased in parallel to the increase of growth inhibitory activity except in the case of analogue G which showed high anti-

Table 2

Circular dichroism of magainins and representative analogues

Peptides	% structure calculated from CD spectra			
	50 mM K-phosphate, pH 7		40% CF_3CH_2OH	
	α-helix	β-sheet	α-helix	β-sheet
Magainin 1	0	44	24	37
Magainin 2	0	46	26	38
B	2	44	61	9
C	0	48	61	14
F	1	46	63	6
G	2	43	52	17

Table 3

Antimicrobial activity of magainin analogues

Organism (ATCC no.)	50% and 100%[a] minimal inhibitory concentration (μg/ml)									
	Magainins		Analogues							
	1	2	A	B	C	D	E	F	G	H
Escherichia coli	100	50	1.2	1.2	10	2.5	1.2	1.2	2.5	70
(25922)	250	100	2.5	2.5	25	5	5	5	5	>100
Klebsiella pneumoniae	100	100	5	5	60	10	5	5	10	100
(13883)	250	100	25	10	100	25	25	25	25	>100
Pseudomonas aeruginosa	430	>100	15	15	>100	25	25	25	25	100
(27853)	>500	>100	25	25	>100	50	50	50	100	>100
Streptococcus agalactiae	60	50	0.25	0.25	0.5	0.25	0.25	0.25	0.5	100
(12386)	75	100	1	1	1	1	1	1	2.5	>100
Streptococcus faecalis	360	>100	5	5	5	5	10	10	10	>100
(212)	>500	>100	10	10	25	25	25	25	50	>100
Staphylococcus aureus	500	>100	5	5	7.5	2.5	5	5	5	>100
(29213) β-lactamase [+]	>500	>100	10	10	25	5	10	10	10	>100
Staphylococcus aureus	360	>100	5	5	10	10	10	10	10	>100
(25923) β-lactamase [−]	>500	>100	10	10	25	25	25	25	25	>100

[a] Minimal level of 100% inhibition is indicated in italics

Magainins 1 and 2 are respectively synthetic. (>) Denotes no activity detected at the concentration indicated

microbial activity but exceptionally low hemolytic activity.

3.4. *Structure and activities*

Omission of replacement at Ser[8] reduced the antimicrobial activity of analogues E, F, and G only slightly. These derivatives (F and G) also displayed, as mentioned above, a lower hemolytic activity which also increases their potential therapeutic value. Acetylation of the α-amino terminus (analogue C) significantly reduced the growth inhibitory activity toward Gram-positive bacteria but the reduction toward Gram-negative bacteria (table 3) was less despite the observation that the proportions of α-helical and apparent β-sheet structures were identical to its non-acetylated counterpart (analogue B). Elongation or substitution with β-alanine at the amino-terminus had no effect on either potency or specificity. These findings suggest that not only enhancement of the α-helical structure is essential but also a free α-amino terminus is required to elicit the maximal antimicrobial activity.

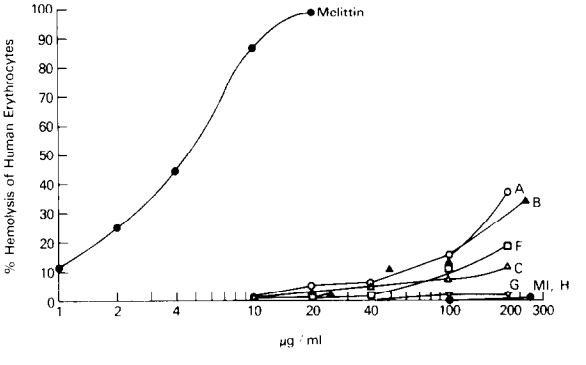

Fig.2. Hemolytic activity of melittin, magainin 1 and analogues.

4. DISCUSSION

Both natural [1] and synthetic [2] magainin peptides have been reported to be broad spectrum antimicrobial substances and to induce osmotic lysis of protozoa. Although the mechanism of action is not yet known, the potential of the magainin peptides to form an amphiphilic helical structure and their broad spectrum of activity led to the speculation that the peptides act on membrane function [1]. The importance of amphiphilic helices has been im-

plicated in a wide variety of biologically active polypeptides whose function is related to the binding of lipids and cell membranes. Recent studies of the correlation of amphiphilic α-helical properties of apolipoproteins [6], melittin [7] and calcitonin [8] with their biological activities support this idea.

In this report, we have shown that magainin peptides do not form α-helical structures in an aqueous buffer. Upon addition of a hydrophobic solvent, the peptides form α-helices. A recent study of the magainin 2 amide analogue using two-dimensional NMR spectroscopy [13] described the same finding. Replacements of low propensity amino acid residues with a high propensity Ala increase not only the α-helical contents, but also antimicrobial activities by one to two orders of magnitude. It should be pointed out that the 100% minimal inhibitory concentration was employed in this study which is different from the previous study [2] in which the peptide concentration causing any growth suppression was considered as the end point. The fact that doubling of helix contents induced by 40% trifluoroethanol causes this remarkable increase in the antimicrobial activity is intriguing. In the modification studies of calcitonin [8], although helical content was only one of the changed variables, an approximate doubling of helical content in either water of 50% aqueous trifluoroethanol resulted in a 2- to 3-fold increase in activity. Similar increases were obtained with corticotropin releasing hormone [14] though the degree of increase in helical content was not stated. NMR or Raman spectroscopic studies of the peptide-lipid interaction may clarify this question.

Substitution of helix promoting residues at Gly^{13} and Gly^{18} seems to be the most important modification for increase in antimicrobial activity. Further substitution at Ser^{8}, although it does not increase helical content or antimicrobial activity, does seem to increase hemolytic activity. Whether the magainin peptides can act through a signal transduction is not known. However, the Ser^{8} and Ser^{23} are potential sites for phosphorylation. It has been observed that replacement of Ser^{8} with Ala in this study and the removal of Ser^{23} [2] has little effect on antimicrobial activity suggesting that phosphorylation at these positions is not involved in the mechanism of action. The considerably lower hemolytic activity of the Ser^{8} analogues may however indicate a detoxification mechanism available to mammalian cells through phosphorylation at this residue. Although acetylation of the amino-terminus generally favors stabilization of α-helical structure, the magainin analogue with acetylated amino-terminus displayed reduced antimicrobial activity suggesting that a free α-amino group providing a positive charge appears to be required to maximize the activity.

In conclusion, this new series of analogues appears to be useful in the study of the structure-function relationship of magainins. In particular, studies of specificity toward various types of membrane by physico-chemical and biological approaches are warranted. Because of the high antimicrobial potency demonstrated in the analogues, they should have a great value in the treatment of bacterial and fungal infections in man and domestic animals.

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