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Augmentation of the Antibacterial Activity of Magainin by Positive-Charge Chain Extension

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Novel analogs of the broad-spectrum antimicrobial peptide magainin-2 were obtained by extension of its chain through addition of segments of positively charged amino acids to either its N or its C terminus and by increasing its helicity. The activity of magainin-2 toward American Type Culture Collection strains of *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* was most considerably enhanced by these modifications, whereas, in general, its low hemolytic capacity was not or was only slightly affected. The antibacterial potencies of magainin-2 and its derivatives were more evident following decreases of pH from 7.2 to 6 and 5.

Magainins are potent antimicrobial basic peptides of 23 amino acid residues, isolated from the skin of the African frog *Xenopus laevis*. They possess a broad range of antibiotic activities against gram-positive and gram-negative bacteria, fungi, and protozoa (1, 3, 19), as well as antiviral capacities (20). The peptides pertain presumably to the immune-defense array of the frog (19, 21).

The mode of action of magainins on microorganisms seems to be complex and, as yet, not entirely clear. As they are highly positively charged peptides, it is likely that they initially interact electrostatically with negative charges that are contained in molecules like lipopolysaccharides and teichoic acids on the target cell surface. At this stage, the peptides apparently have random structures. Following attachment to the cells, the peptides assume, through hydrophobic interactions, an amphiphilic α-helical structure that is probably organized in a multimeric form, allowing the formation of ion channels and perturbation of bacterial membranes (1, 3).

In light of the significant therapeutic potential of magainins as antimicrobial drugs, rather intensive structure-function studies of the peptides were performed (4, 5). These studies aimed at both the design and the preparation of superactive derivatives, as well as at understanding their physicochemical features and modes of action. The studies explicitly indicate that the enhancement of the α-helical nature of magainin parallels augmentation of antibiotic activity (1, 4).

In the present study, we chose to evaluate the influence of the addition of a set of positively charged lysine and arginine residues to the carboxylic and amino termini of magainin-2 on its antibacterial activity. Our assumption was that these cationic amino acid moieties would enhance the helicity (14) as well as strengthen the interaction between magainin-2 and the negatively charged bacterial surface with a consequent augmentation of membrane perturbation. We also prepared analogs of magainin-2, with the aim of increasing α-helical and amphiphilic tendencies. Indeed, the antibacterial activities of several analogs were most significantly increased, whereas the minute hemolytic capacity of the parent peptide, i.e., magainin-2, remained unchanged.

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MATERIALS AND METHODS

Peptide synthesis. Synthesis of magainin-2 and its different analogs was carried out by the solid-phase strategy (9). Peptide chains were assembled manually on a chloromethylated polystyrene–2% divinylbenzene resin (Chemalog, South Plainfield, N.J.). Protected amino acid derivatives were purchased from Bachem (Bubendorf, Switzerland). α-Amino groups of amino acids were protected by the *t*-butyloxycarbonyl moiety. Side-chain-protecting groups were as follows: serine, *O*-benzyl; glutamic acid, γ-benzyl; lysine, N^{ε}-2-chlorobenzyloxycarbonyl; histidine, N^{im}-benzyloxycarbonyl; and arginine, N^{ω}-tosyl. All coupling stages were performed with a threefold excess of protected amino acid derivatives with an equimolar mixture of *N*,*N*′-dicyclohexylcarbodiimide and 1-hydroxybenzotriazole (Aldrich, Milwaukee, Wis.) as reagents. Deprotection of peptides and cleavage from resin were achieved by an anhydrous HF procedure (15). Crude peptides were purified to homogeneity by initial chromatography on a Sephadex G-15 column with 0.1 N acetic acid as an eluent, followed by preparative high-performance liquid chromatography (HPLC) on a LiChrosorb RP-8 column (240 by 10 mm; diameter, 7 μm) (Merck, Darmstadt, Germany) with a linear gradient of acetonitrile (10 to 60%) in 0.1% aqueous trifluoroacetic acid. Amino acid analysis was performed on a Dionex automatic amino acid analyzer. Sequence determination was accomplished with an Applied Biosystems 470A gas-phase microsequencer hooked to an Applied Biosystems 120A PTH analyzer.

Circular dichroism studies. Far-UV circular dichroism spectra of synthetic compounds were examined. The peptides (0.25×10^{-5} M to 1×10^{-5} M) were each dissolved in either 50 mM potassium phosphate buffer (pH 7.0) or 50% (vol/vol) trifluoroethanol (Merck) in the same buffer. A quartz cell with a 1.0-cm pathlength was employed. Scans were performed with a Jasco model J-500C spectrophotometer with a Jasco NP-500 data processor, at room temperature over a wavelength range of 240 to 200 nm. Calculations of α-helix and β-sheet contents were performed as described elsewhere (13).

Antibacterial studies. Stock solutions of magainin-2 and

Peptides	Sequence
1 (Mag.2)	NH_2-Gly-Ile-Gly-Lys-Phe-Leu-His-Ser-Ala-Lys-Lys-Phe-Gly-Lys-Ala-Phe-Val-Gly-Glu-Ile-Met-Asn-Ser-OH
2	NH_2-$(Lys)_4$-Gly - Ser-OH
3	NH_2-$(Lys)_{10}$-Gly - Ser-OH
4	NH_2-$(Ala)_{10}$-Gly - Ser-OH
5	NH_2-$(Arg)_{10}$-Gly - Ser-OH
6	NH_2-$(Lys)_{20}$-Gly - Ser-OH
7	NH_2-Gly - Ser-$(Lys)_{10}$-OH
8	NH_2-Lys-Lys-Phe-Leu-His-Ser-Ala-Lys-Lys-Trp-Leu-Lys-Ala-Phe-Val-Lys-Leu-Phe-Lys-Asn-Trp-OH
9	NH_2-Gly-Ile-Gly-Lys-Leu-Phe-Leu-His-Ala-Ala-Lys-Lys-Phe-Ala-Lys-Ala-Phe-Val-Ala-Glu-Lys-Met-Asn-Ser-OH
10	NH_2-$(Lys)_{10}$-Gly-Ile-Gly-Lys-Leu-Phe-Leu-His-Ala-Ala-Lys-Lys-Phe-Ala-Lys-Ala-Phe-Val-Ala-Glu-Lys-Met-Asn-Ser-OH

FIG. 1. Sequences of magainin-2 (Mag. 2) and analogs.

analogs were prepared from pure peptide powders. Compounds were dissolved in water, filter sterilized (0.45-μm-pore-size Acrodisc filter; Gelman Sciences), and stored at −70°C. No change in antimicrobial activity was detected in any compound over a 1-month period. Chromatographic controls revealed that all of the peptides studied proved to be chemically stable at corresponding experimental conditions.

The in vitro antimicrobial activities of magainin-2 and the various analogs were tested by the microdilution technique with tryptic soy broth (Difco Laboratories, Detroit, Mich.) used as a growth medium. The bacteria isolated included American Type Culture Collection (ATCC) strains of *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The bacterial inoculum was 5×10^5 CFU/ml, and microplates were incubated at 37°C for 18 to 20 h.

MBC assays were performed as described elsewhere (17). Briefly, from each corresponding clear well of MIC microbroth plate incubated overnight at 37°C, 10 μl was removed with a multipoint inoculator and spotted on blood agar plates. The MBC endpoint was the concentration of compound at which no growth (i.e., 99.9% kill) occurred after 20 h of incubation.

The in vitro activities of the different analogs at various pH conditions were studied by the broth microdilution method in tryptose broth (Difco) with adjusted pH values in the range of 5.0 to 8.0, with *E. coli* and *P. aeruginosa* as test organisms.

The effect of human serum on the antibacterial activity of magainin-2 and analogs was tested on *P. aeruginosa* by the microdilution method with a medium containing a 1:1 ratio of tryptic soy broth and pooled human serum, at 37°C for 18 to 20 h.

In each of these tests, the MICs and MBCs of the various analogs were compared with the activity of magainin-2.

Hemolytic assay. The hemolytic capacities of the various synthetic peptides were evaluated with human erythrocytes essentially as described elsewhere (2). Briefly, packed cells (3 ml) were washed three times with isotonic phosphate-buffered saline (PBS) (pH 7.4) and diluted to a final volume of 20 ml with the same buffer. Aliquots (190 μl) of cell suspension were placed in Eppendorf tubes, and solutions (10 μl) of different concentrations of the tested peptides, in PBS, were added. Following gentle mixing for 30 min at 37°C, the tubes were centrifuged at 4,000 × *g* for 5 min. Aliquots (100 μl) of supernatants were taken and diluted to 1 ml with PBS, and A_{576} was measured. The level of hemolysis caused by 0.1% Triton X-100 was considered 100%.

RESULTS

Preparation and characterization of magainin-2 and analogs. The various peptides prepared for the present study are listed in Fig. 1. The crude compounds, obtained in solid-phase synthesis, were purified to homogeneity by HPLC, and their correct compositions and primary structures were ascertained by amino acid and sequence (for peptides 1 and 7 to 9) analyses, respectively. Some parameters related to the secondary structures of the synthetic peptides assessed by circular dichroism measurements are summarized in Table 1. The data indicate that in aqueous phosphate buffer, the peptides are not α-helical and contain rather distinct β-sheet conformations. In the presence of 50% trifluoroethanol, however, the α-helical structures are dramatically formed. It is clear that chain extensions of magainin-2 or within-chain modifications promote these structural alterations. Thus, whereas magainin-2 demonstrates 35% helicity, this feature is increased, for example, to 62 or 88% when

TABLE 1. Structural data from circular dichroism of magainin-2 and analogs

Peptide no.[a]	% of secondary structure in the presence of:			
	50 mM potassium phosphate (pH 7)		50% CF_3CH_2OH[b]	
	α-Helix	β-Sheet	α-Helix	β-Sheet
1	0	79	35	26
2	0	60	67	28
3	0	53	62	38
4			59	30
5			45	20
6	0	56	44	38
7	0	38	88	12
9	0	57	56	18

[a] Peptide 1, magainin-2; peptides 2 through 9, analogs of magainin-2.
[b] In phosphate buffer (pH 7).

TABLE 2. In vitro susceptibilities of ATCC strains of *E. coli*, *P. aeruginosa*, and *S. aureus* to magainin-2 and analogs

Peptide no.[a]	MIC/MBC (μg/ml)		
	E. coli ATCC 25922	*P. aeruginosa* ATCC 27853	*S. aureus* ATCC 29213
1	50/100	50/100	≥100
2	≥100	100/100	≥100
3	6.25/6.25	6.25/12.5	12.5/50
4	>400	>400	>400
5	3.12/6.25	6.25/6.25	25/25
6	12.5/12.5	6.25/12.5	25/50
7	50/≥100	3.1/6.25	3.1/6.25
8	12.5/100	25/100	25/50
9	12.5/25	6.25/25	100/100
10	12.5/25	6.25/25	25/50

[a] Peptide 1, magainin-2; peptides 2 through 10, analogs of magainin-2.

TABLE 3. Effect of pH on the in vitro activities of magainin-2 and its analogs against *P. aeruginosa* ATCC 27853

Peptide no.[a]	MIC/MBC (μg/ml) with tryptose broth at the following pH:		
	6.0	7.2	8.0
1	1.57/1.57	50/50	>50/>50
2	3.12/3.12	>50/>50	>50/>50
3	≤0.78/≤0.78	3.12/6.25	≤0.78/12.5
6	1.57/1.57	6.25/6.25	6.25/6.25
7	≤0.78/≤0.78	1.57/1.57	3.12/25
8	≤0.78/≤0.78	3.12/3.12	12.5/12.5
9	1.57/1.57	6.25/6.25	25/50
10	1.57/1.57	3.12/3.12	25/25

[a] Peptide 1, magainin-2; peptides 2 through 10, analogs of magainin-2.

a segment of 10 lysine residues (Lys_{10}) is added to its N (peptide 3) or C (peptide 7) terminus, respectively.

Antibacterial activity. Addition of a set of positively charged amino acids, i.e., lysine and arginine, at either the amino or the carboxyl terminus of magainin-2 improves its antibacterial activity against three ATCC strains of *E. coli*, *P. aeruginosa*, and *S. aureus* (Table 2). The length of the positive stretch seems, however, to be of great importance. Thus, although the addition of 10 or 20 lysine residues is very effective (with the sole exception of peptide 7 against *E. coli*), a set of only 4 lysines added at the amino terminus of magainin-2 (peptide 2) surprisingly decreases the activity somewhat. As expected, Arg_{10}–magainin-2 (peptide 5) and Lys_{10}–magainin-2 (peptide 3) possess similar antibacterial potencies. Both arginine and lysine are equally and fully protonized at pH 7.2. As shown in Table 2, chain extension by 10 residues of alanine yielded an inactive derivative (peptide 4).

Peptide 8 was synthesized with the aim of enhancing the amphiphilic organization of magainin-2 by means of replacing certain amino acid residues with stronger hydrophobic and hydrophilic residues (Fig. 2). α-Helical wheel projection (14) revealed that, indeed, amphiphilicity was augmented by these modifications. This peptide exhibited a significant increase of activity compared with magainin-2 (Table 2). Its hemolytic activity, however, was dramatically increased (see Table 6). Peptide 8 is composed of 21 amino acid residues. It was reported that shortening of the magainin-2 chain (23-mer) by removal of the first two amino acids from its N terminus does not affect antibacterial potency (21). Peptide 9, in which enhancement of α-helicity was intended, showed increased activity toward *E. coli* and *P. aeruginosa* cells (Table 2). Addition of 10 lysine residues to its amino terminus (peptide 10) further augmented activity toward *S. aureus* (Table 2). Tables 3 and 4 summarize the effects of different pH values on the in vitro activities of the synthetic peptides in tryptose broth against *P. aeruginosa* and *E. coli*, respectively. As shown in Tables 3 and 4, the peptides (including magainin-2) are generally more potent at pH values lower than 7.2. It has already been reported (12) that *E. coli* can grow at a pH range of 4.4 to 9.0. It is worth mentioning that although the growth curves might vary within these values, our control studies at pH 5.0 and 8.0 (20 h, 37°C) revealed that the final CFU counts did not differ significantly from those at pH 7.2.

The effects of pooled human serum on the in vitro activities of magainin-2 and its analogs against *P. aeruginosa* are summarized in Table 5. As shown, addition of 50% serum resulted in a two- to eightfold reduction in antipseudomonal activity of all peptides tested. The serum was added immediately before the assay was performed. Control studies demonstrated that following 20 h of incubation, no differences between final CFU values with or without serum could be detected. The pH of the mixture did not change significantly (less than 0.4 to 0.5 pH units) during the incubation period.

Hemolytic activity. Table 6 illustrates that, with the exceptions of peptide 8, peptide 6 (Lys_{20}–magainin-2), and peptide 4 (Ala_{20}–magainin-2), all of the peptides studied, including magainin-2, are nonhemolytic at concentrations of up to 200 μg/ml. The concentration of erythrocytes used in our in vitro hemolytic assays is rather close to the actual physiological levels of erythrocytes in humans. This fact may hint that nonhemolytic peptides will be harmless to cells in vivo.

DISCUSSION

The data presented demonstrate that extending the chain of magainin by adding positively charged amino acids at either terminus results in severalfold enhancement of its antibacterial activity (Table 2). With the aim of examining whether this stimulation stems primarily from the net charge, is due to enhancement of α-helicity, or is derived for both reasons, the peptide Ala_{10}–magainin-2 (peptide 4) (Fig. 1) was synthesized. As expected, its α-helicity was significantly higher than that of magainin-2 (Table 1). It was, however, devoid of any antibacterial activity at concentrations up to 400 μg/ml. The explanation for this finding is not

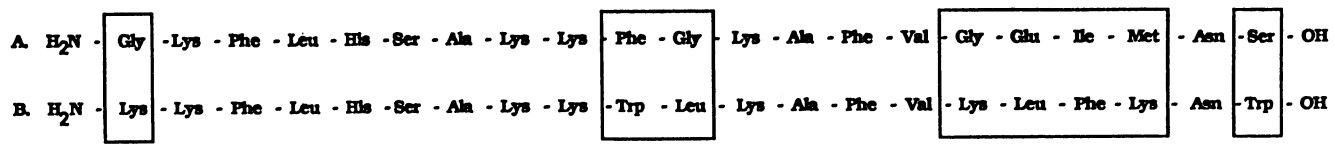

FIG. 2. Sequences of des-Gly-Ile-magainin-2 (A) and peptide 8 (B). Amino acid substitutions are framed.

TABLE 4. Effect of pH on the in vitro activities of magainin-2 and its analogs against *E. coli* ATCC 25922

Peptide no.[a]	MIC/MBC (μg/ml) with tryptose broth at the following pH:			
	5.0	6.0	7.2	8.0
1	12.5/12.5	25/25	50/50	50/50
2	25/25	≥100/≥100	100/100	≥100/≥100
3	≤0.78/≤0.78	≤0.78/≤0.78	1.57/1.57	3.12/3.12
6	≤0.78/≤0.78	3.12/3.12	6.25/6.25	12.5/12.5
7	12.5/25	50/50	25/25	25/>100
8	≤0.78/≤0.78	1.57/1.57	3.12/3.12	6.25/25
9	≤0.78/1.57	3.12/3.12	3.12/3.12	6.25/6.25
10	≤0.78/1.57	3.12/3.12	3.12/3.12	3.12/3.12

[a] Peptide 1, magainin-2; peptides 2 through 10, analogs of magainin-2.

straightforward. It is possible that the alanine chain prevents the self-assembly of magainin needed for expression of activity (1). Alternatively, the oligoalanine stretch may impair the interaction of the magainin moiety with the cell surface. In view of these possibilities, however, it seems that amplification of the net positive charge of magainin-2 plays a decisive role in augmenting antimicrobial activity. Furthermore, the number of positive charges is most significant, since the addition of four lysines to magainin did not practically alter its activity, while the addition of 10 to 20 residues led to a major increase in activity. It should be emphasized that the implications of structural features, i.e., helicity, amphiphilicity, and overall positive nature, for the antibacterial capacities of magainin and its derivatives are rather indirect. This results from the fact that information concerning the actual structures of the peptides, in terms of their association with bacteria and their exact modes of action, is not yet available.

Synthetic cationic poly- and copoly-α-amino acids have been shown to be lethal and cytotoxic for different bacteria, as well as toward mammalian cells (6, 8). Polycationic molecules, such as protamine and oligolysines (e.g., Lys_{20}) were reported to disorganize the bacterial outer membrane through their interaction with surface lipopolysaccharides (18). We have thus examined the antibacterial capacity of Lys_{17} in the presence and absence of magainin-2. The oligolysine was found to be less active than magainin, and their coapplication did not affect the antimicrobial activity of magainin. The extended, positively charged magainin-2 derivatives, however, exhibited intramolecular synergism of their two constituents; i.e., when the two peptidic moieties are linked covalently to each other, a synergistic antibacterial effect is observed. Two types of surface-active peptides are worth mentioning here, namely, melittin and sarcotoxins. Melittin, isolated from bee venom (7), is a potent hemolytic and antirheumatic peptide, entailing a positively charged C terminus head of lysine and arginine residues. Sarcotoxins (11), isolated from the fresh fly *Sarcophaga peregrina*, contain a similar positive N terminus head. These latter peptides are potent bactericidal agents. It is not yet clear why our extended magainin derivatives, Lys_{10}- and Arg_{10}-magainin-2, and sarcotoxins (10) are nontoxic to human erythrocytes, whereas melittin is highly hemolytic. However, it is worth noting that, as expected (16), when the number of lysine residues added to magainin is increased from 10 to 20, hemolytic activity begins to be apparent.

TABLE 5. Influence of human serum on the in vitro activities of magainin-2 and its analogs against *P. aeruginosa* ATCC 27853

Peptide no.[a]	MIC/MBC (μg/ml) in the presence of:	
	Tryptose soy broth (100%)	Tryptose soy broth (50% human serum)
1	50/100	100/100
2	>100/>100	>100/>100
3	6.25/12.5	25/50
6	6.25/12.5	50/>100
7	3.12/6.25	25/50
8	25/100	>100/>100
9	6.25/25	25/50
10	6.25/25	>100/>100

[a] Peptide 1, magainin-2; peptides 2 through 10, analogs of magainin-2.

TABLE 6. Percent hemolytic activities of magainin-2 and its analogs

Peptide no.[a]	% Hemolytic activity at the following peptide concns (μg/ml)[b]			
	25	50	100	200
1	0	0	0	1
3	0	0	0	0
4	0	0	2	9
5	0	1	2	4
6	1	2	6	14
7	0	0	0	1
8	3	16	29	41
9	0	0	0	0
10	0	0	0	2

[a] Peptide 1, magainin-2; peptides 2 through 10, analogs of magainin-2.
[b] The level of hemolysis obtained with Triton X-100 was considered 100%.

It is also of interest that magainin-2 and its analogs displayed enhanced in vitro activities against *E. coli* and *P. aeruginosa* at relatively low pH values, whereas common classes of antimicrobial agents such as aminoglycosides and quinolones are usually less active at reduced pH values. The relatively high bactericidal activity at low pH values is of potential importance, since in many sites of infections, such as abscesses, acidic pH is apparent. The increase of the potency of magainin-2 and its analogs at relatively low pH values may stem from structural alterations and their consequent implications to perturbation of the bacterial cell membrane, i.e., the change in pH affects the mode of peptide-cell interaction. However, this assumption needs further substantiation.

The possibility that magainin-2 and related derivatives will become antibacterial agents for systemic use is doubtful. Their topical application either in the treatment of wound infections and burns or for inhalation therapy in cystic fibrosis patients, however, may be realized. Toward this goal, we and others are directing further efforts at synthesis.

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