A Repertoire of Novel Antibacterial Diastereomeric Peptides with Selective Cytolytic Activity*

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The increase in infectious diseases and bacterial resistance to antibiotics has resulted in intensive studies focusing on the use of linear, \(\alpha\)-helical, cytolytic peptides from insects and mammals as potential drugs for new target sites in bacteria. Recent studies with diastereomers of the highly potent cytolytic peptides, pardaxin and melittin, indicate that \(\alpha\)-helical structure is required for mammalian cells lysis but is not necessary for antibacterial activity. Thus, hydrophobicity and net positive charge of the polypeptide might confer selective antibacterial lytic activity. To test this hypothesis, a series of diastereomeric model peptides (12 amino acids long) composed of varying ratios of leucine and lysine were synthesized, and their structure and biological function were investigated. Peptide length and the position of \(\alpha\)-amino acids were such that short peptides with stretches of only 1–3 consecutive \(\alpha\)-amino acids that cannot form an \(\alpha\)-helical structure were constructed. Circular dichroism spectroscopy showed that the peptides do not retain any detectable secondary structure in a hydrophobic environment. This enabled examination of the sole effect of hydrophobicity and positive charge on activity. The data reveal that modulating hydrophobicity and positive charge is sufficient to confer antibacterial activity and cell selectivity. A highly hydrophobic diastereomer that permeated both zwitterionic and negatively charged phospholipid vesicles, lysed eukaryotic and prokaryotic cells. In contrast, a highly positively charged diastereomer that only permeated slightly negatively charged phospholipid vesicles had low antibacterial activity and could not lyse eukaryotic cells. In the boundary between high hydrophobicity and high positive charge, the diastereomers acquired selective and potent antibacterial activity. Furthermore, they were completely resistant to human serum inactivation, which dramatically reduces the activity of native antibacterial peptides. In addition, a strong synergistic effect was observed with the inactivation of the peptides with the antibiotic tetracycline on resistant bacteria. The results are discussed in terms of proposed mechanisms of antibacterial activity, as well as a new strategy for the design of a repertoire of short, simple, and easily manipulated antibacterial peptides as potential drugs in the treatment of infectious diseases.

Infectious diseases are increasing phenomena today mainly as a result of changes in the spectrum of pathogens and the increase in antibiotic-resistant pathogens. When antibiotics were first introduced, the proportion of resistant versus nonresistant pathogens was less than 1%, but this percentage has dramatically increased within 12 years of their uses. Although the number of antibacterial products available today is impressive (about 150), they cover only six different target sites and are dominated by \(\beta\)-lactams, tetracyclines, aminoglycosides, marcolides, sulfonamides, and quinolones (1). The search for new target sites has resulted in an increased interest in antibacterial peptides that could serve as potential therapeutic drugs. Antibacterial peptides were initially discovered in invertebrates (2, 3) and subsequently in vertebrates, including humans (4). Antibacterial peptides serve as a defense system in addition or complementary to the highly specific cell-mediated immune response. This secondary, chemical immune system provides organisms with a repertoire of small peptides that are synthesized promptly upon induction and that act against invasion by occasional and obligate pathogens as well as against the uncontrolled proliferation of commensal microorganisms (5–7). So far, more than 100 different antibacterial peptides have been isolated and characterized. Most of them appear to act by direct lysis of the pathogenic cell membrane. A major group within this family are short linear polypeptides (\(\leq40\) amino acids), which are devoid of disulfide bridges (6). These polypeptides vary considerably in chain length, hydrophobicity, and overall charge distribution but share a common structure upon association with lipid bilayers, namely, an amphipathic \(\alpha\)-helical structure (8). Unlike bee venom melittin (9) and the neurotoxin pardaxin (10, 11) that are cytotoxic to both bacteria and mammalian cells (12, 13), antibacterial peptides are active only against bacteria. These peptides include the cecropins isolated from the cecropia moth (2), the magainins (3), and dermaseptins (14) isolated from frogs skin.

Numerous studies conducted on various native antibacterial peptides tend to emphasize the importance of an amphipathic \(\alpha\)-helical structure and a net positive charge for cytolytic activity. The positive charge facilitates peptides interaction with the negatively charged membranes (15–18) found in higher concentrations in the pathogenic cell membrane as compared with normal eukaryotic cells, and the amphipathic \(\alpha\)-helical structure is essential for lytic activity (18, 19). Studies using model antibacterial peptides focused on chain length and amino acid composition, as well as amphipathic structure (20–24). Most of these studies simultaneously examined at least three parameters, making it difficult to distinguish between the effect of each individual parameter on the overall biological activity. Nevertheless, these studies proved that an amphipathic \(\alpha\)-helix and a net positive charge are required for antibacterial activity.

In previous studies, \(\beta\)-amino acids were incorporated into the cytolytic peptides pardaxin (25) and melittin (26). The resulting diastereomers did not retain their \(\alpha\)-helical structure, which caused abrogation of their cytotoxic effects on mammalian...
cells. However, the diastereomers retained high antibacterial activity. These results suggest that hydrophobicity and a net positive charge confer selective antibacterial activity to non-lective cytolytic peptides and that amphipathic α-helical structure is not required. However, the diastereomers of pardaxin and melittin contained long stretches of l-amino acids (14–17 amino acids long), which raises the possibility that the low residual helicity could be sufficient for membrane binding and destabilization.

To examine whether modulating hydrophobicity and the net positive charge of linear cytotactic polypeptides is sufficient to confer selective antibacterial activity, we chose to investigate diastereomers of short model peptides (12 amino acids long), composed of varying ratios of leucine and lysine and one third of their sequence composed of n-amino acids. Peptide length and the position of d-amino acids were such that short peptides with very short consecutive stretches of 1–3 l-amino acids that cannot form an α-helical structure were constructed. The diastereomers were evaluated with regard to (1) their cytotoxicity against bacteria and human erythrocytes, (2) their structure, and (3) their ability to interact and perturb the morphology of the bacterial wall and model phospholipid membranes. The data show that modulating hydrophobicity and positive charge is sufficient to confer antibacterial activity and cytolitic selectivity. Furthermore, the resulting antibacterial peptides act synergistically at nonlethal concentrations with antibacterial drugs such as tetracycline, and they are totally resistant to human serum inactivation that dramatically reduces the activity of native antibacterial peptides. The results are discussed in light of proposed mechanisms of antibacterial activity, as well as a new strategy for the design of a repertoire of short, simple, and easily manipulated antibacterial peptides as potential drugs in the treatment of infectious diseases.

**EXPERIMENTAL PROCEDURES**

**Materials**—Butyloxy-carbonyl-(amino acid)-(phenyl-acetamido)methyl resins were prepared from Applied Biosystems (Foster City, CA), and butyloxy-carbonyl amino acids were obtained from Peninsula Laboratories (Belmont, CA). Other reagents used for peptide synthesis included trifluoroacetic acid (Sigma), N,N-diisopropyl-carbamoyl (Aldrich, dis-tilled over ninhydrin), di-cyclohexyl-carbodiimide (Fluka), 1-hydrox-ybenzotriazole (Pierce), and dimethylformamide (peptide synthesis grade, BioJet). Egg phosphatidylcholine (PC)1 was purchased from Lipid Prod-ucts (South Nutfield, UK). Egg phosphatidylglycerol (PG) and phos-phatidylethanolamine (PE) (Type V, from Escherichia coli) were pur-chased from Sigma. Cholesterol (extra pure) was supplied by Merck (Darmstadt, Germany) and recrystallized twice from ethanol. 3,3′-di-ethylthio-di-carboxyamine iodide (dis-C5–S) was obtained from Molecular Probes (Eugene, OR). All other reagents were of analytical grade. Buff-ers were prepared in double glass-distilled water.

**Peptide Synthesis and Purification**—Peptides were synthesized by a solid phase method on butyloxy-carbonyl-(amino acid)-(phenylac-eamido) methyl resin (0.05 meq) (27). The resin-bound peptides were cleaved from the resins by hydrogen fluoride and after hydrogen fluo-ride evaporation extracted with dry ether. These crude peptide prepa-rations contained one major peak, as revealed by reversed phase HPLC, that was 50–70% pure peptide by weight. The synthesized peptides were further purified by reversed phase HPLC on a C18 reversed phase Bio-Rad semi-preparative column (250 × 10 mm, 300 Å pore size, 5 μm particle size). The column was eluted in 40 min, using a linear gradient of 5–60% acetonitrile in water, both containing 0.05% trifluoroacetic acid (v/v), at a flow rate of 1.5 ml/min. The purified peptides, which were shown to be homogeneous (95%) by analytical HPLC, were subjected to amino acid analysis and mass spectroscopy to confirm their composition.

**Transamination of the Peptides**—Resin-bound peptide (20 mg) was treated for 3 days with a mixture composed of saturated ammonia solution (30%) in methanol and MeSO (1:1 v/v), which resulted in transamination of the carboxylate group of the lysine residue located at the C terminus of all analogues. Thus, peptides were obtained in which all the protecting groups remained attached, but whose terminal residues were modified by the side group. The ethanol and threo-monia were evaporated under a stream of nitrogen, and the MeSO was evaporated by lyophilization. The resulting protected peptides were then extracted from the resin with fresh MeSO and precipitated with dry ether. The products were subjected to hydrogen fluoride cleavage and further purified by reversed phase HPLC as described above.

**Preparation of Serum**—Blood was collected from five volunteers and allowed to clot at room temperature for 4 h. The blood was then cen-trifuged for 15 min at 1500 × g, and the serum was removed and pooled. The serum complement was inactivated by heating at 56 °C for 30 min.

**CD Spectroscopy**—The CD spectra of the peptides were measured with a Jasco J-500A spectropolarimeter. The spectra were scanned at 23 °C in a capped, quartz optical cell with a 0.5-mm path length. Spectra were obtained at wavelengths of 250–190 nm. Eight scans were taken for each peptide at a scan rate of 20 nm/min. The peptides were scanned at concentrations of 1.5 × 10^{-5}–2.0 × 10^{-5} M in 40% trifluoro-ethanol. Fractional helicities (30, 31) were calculated as follows:

\[ f_\theta = \frac{[\theta]_{222}^\text{obs}}{[\theta]_{222}^\text{calc}} - 1 \]  

where \([\theta]_{222}^\text{obs}\) is the experimentally observed absolute mean residue ellipticity at 222 nm, and values for \([\theta]_{222}^\text{calc}\) corresponding to 0 and 100% helix content at 222 nm, estimated at 2,000 and 32,000 degrees cm²dor mol, respectively.

**Antibacterial Activity of Diastereomeric Model Peptides**—The antibacterial activity of the diastereomers was examined in sterile 96-well plates (Nunc F96 microtiter plates) in a final volume of 100 μl as follows. Aliquots (50 μl) of a suspension containing bacteria at a concentra-tion of 10^6 colony-forming units/ml LB medium were added to 50 μl of water or 60% pooled normal human serum in PBS, containing the peptide in 2-fold serial dilutions. Growth inhibition was determined by measuring the absorbance at 492 nm with a Microplate autoreader El309 (Bio-tek Instruments), following incubation for 18–20 h at 37 °C. Antibacterial activity is expressed as the minimal inhibitory concentra-tion (MIC), the concentration at which 100% inhibition of growth was observed after 18–20 h of incubation. The bacteria used were: E. coli D21, Acinetobacter calcoaceticus ATCC 17940, Pseudomonas aeruginosa ATCC 27853, Bacillus megaterium Bn1, and Bacillus subtilis ATCC 6051.

**Synergistic Effect between Tetracycline and the Diastereomers**—To investigate a possible synergistic relationship between the antibiotic drug tetracycline and the diastereomers, tetracycline was tested in 2-fold serial dilutions against P. aeruginosa (ATCC 27853) in the presence of a constant equimolar concentration (1 μM) of [D]-L-L-1, 10^{-4}, K_{L_{1}}. Antibacterial activity of the mixtures was determined as described above.

**Hemolysis of Human Red Blood Cells**—Hemolytic activity of the peptides were tested against human red blood cells (hRBC). Fresh hRBC with EDTA were rinsed three times with PBS (35 mM phosphate buffer/0.15 mM NaCl, pH 7.3) by centrifugation for 10 min at 800 × g and resuspended in PBS. Peptide solution in PBS was then added to 50 μl of a solution of the stock hRBC in PBS to reach a final volume of 100 μl (final erythrocyte concentration, 5% v/v). The resulting suspension was incubated under agitation for 30 min at 37 °C. The samples were then centrifuged at 800 × g for 10 min. Release of hemoglobin was monitored by measuring the absorbance of the supernatant at 540 nm. Controls for zero hemolysis (blank) and 100% hemolysis consisted of hRBC suspended in PBS and Triton X-1%, respectively.

1 The abbreviations used are: PC, egg phosphatidylcholine; dis-C5–S, 3,3′-diethylthiodicarboxyamine iodide; hRBC, human red blood cells; MIC, minimal inhibitory concentration; PBS, phosphate-buffered sa-line; PE, phosphatidylethanolamine; PG, egg phosphatidylglycerol; HPLC, high performance liquid chromatography.
Peptides Induced Membrane Permeation—Membrane permeation was assessed utilizing the diffusion potential assay (32, 33) as described previously (34, 35). In a typical experiment, in a glass tube, 4 μl of a liposome suspension (final phospholipid concentration, 32 μM) in a K-containing buffer (50 mM K₃SO₄, 25 mM HEPES-SO₄, pH 6.8), was diluted in 1 ml of an isotonic K⁺ free buffer (50 mM Na₂SO₄, 25 mM HEPES-SO₄, pH 6.8), and the fluorescent, potential-sensitive dye diSC₂(5) was then added. Valinomycin (1 μM, diluted in 1 ml of an isotonic K⁺ containing buffer (50 mM K₂SO₄, 25 mM HEPES-SO₄, pH 6.8), was added to the suspension to slowly create a negative diffusion potential inside the vesicles, which led to a quenching of the dye’s fluorescence. Once the fluorescence had stabilized, which took 3–10 min, peptides were added. The subsequent dissipation of the diffusion potential, as reflected by an increase in fluorescence, was monitored on a Perkin-Elmer LS-50B spectrophotometer, with the excitation set at 620 nm, the emission set at 670 nm, and the gain adjusted to 100%. The percentage of fluorescence recovery, Fᵣ, was defined as:

\[ Fᵣ = \left( I_f - I/I_i - I \right) \times 100 \]  

where \( I_i \) represents the initial fluorescence, \( I_f \) represents the total fluorescence observed before the addition of valinomycin, and \( I \) represents the fluorescence observed after adding the peptide at time \( t \).

Visualization of the Effect of the Peptides on Bacteria Using Electron Microscopy—Samples containing E. coli (10⁶ colony-forming units/ml) in LB medium were incubated with the various peptides at their MIC concentrations. Designations are as follows: [D]-L3,4,8,10-K₄L₈; [D]-L3,4,8,10-K₅L₇; and [D]-L3,4,8,10-K₇L₅.

RESULTS

Diastereomers Design—Four diastereomers of linear and short (12 amino acids long) model peptides composed of varying ratios of lysine-to-leucine were synthesized in order 1) to examine whether a balance between hydrophobicity and a net positive charge may be a sufficient criteria necessary for selecting bacterial lysis and 2) to gain insight into the mechanism underlying this effect. The location of α-amino acids remained constant in all peptides, because it was constructed for maximum disruption of α-helical structure. α-Amino acids were distributed along the peptide, leaving only very short stretches of 1–3 consecutive l-amino acids. The peptides were then characterized with regard to their structure, biological function, and interaction with bacteria and model membranes composed of either zwitterionic or negatively charged phospholipids. The following peptides were synthesized: [D]-L3,4,8,10-K₄L₈, [D]-L3,4,8,10-K₅L₇, [D]-L3,4,8,10-K₇L₅, and [D]-L3,4,8,10-K₁₀ (Table I). The hydrophobicities (36) and net positive charge of the peptides are listed in Table I.

CD Spectroscopy—The extent of the α-helical structure of the diastereomers was determined from their CD spectra in 40% trifluoroethanol, a solvent that strongly promotes α-helical structure. As expected, after incorporation of α-amino acids, no signal was observed for all the diastereomers, demonstrating the lack of any specific secondary structure (data not shown). In a recent study, a peptide with a sequence identical to that of [D]-L3,4,8,10-K₁₀, but composed of only l-amino acids was found to have ~40% α-helical structure in methanol and in 1α-dimyristoylphosphatidylcholine vesicles (22).

Antibacterial and Hemolytic Activity of the Peptides—The hemolytic activity of the peptides against the highly cytolyti-
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Synergistic Effects and Antibacterial Activity of the Diastereomers in Serum—We observed a synergistic effect between the antibiotic drug tetracycline and the diastereomer [D]-L3,4,8,10-K5L7. Tetracycline shows little activity against P. aeruginosa. However, when mixed with 1 μM solution of [D]-L3,4,8,10-K5L7, a concentration that is 10-fold lower than that required for lytic activity against P. aeruginosa, an 8-fold increase in the activity of tetracycline was observed (Table III). A possible explanation for the synergistic effect is that the peptide slightly disrupts the bacterial wall, which improves partitioning of tetracycline into the bacteria. This is supported by electron microscopy studies showing that below its MIC [D]-L3,4,8,10-K5L7 causes morphological changes in the bacterial wall (see Fig. 3). In addition, the effect of pooled human serum on the antibacterial activity of [D]-L3,4,8,10-K5L7 and the native antibacterial peptide dermaseptin against P. aeruginosa and E. coli was found to differ considerably (Table III). Although dermaseptin was 8–10-fold less active in the presence of serum, [D]-L3,4,8,10-K5L7 retained its antibacterial activity.

Peptide-Induced Membrane Permeation—Various concentrations of peptides were mixed with vesicles that had been pre-treated with the fluorescent dye, diS-C2-5, and valinomycin. The kinetics of the fluorescence recovery was monitored, and the maximum fluorescence level was determined as a function of peptide concentration (Fig. 2). PC/cholesterol vesicles (10:1) served as a model of the phospholipid composition of the outer erythrocyte leaflet (37), and PE/PG vesicles (7:3) were used to mimic the phospholipid composition of E. coli (38). We found a direct correlation between the potential of the peptides to permeate PE/PG vesicles and their lytic activity against erythrocytes and E. coli. Only the hemolytic peptide [D]-L3,4,8,10-K5L7 permeated the zwitterionic phospholipid vesicles. Furthermore, the ability of the peptides to permeate PE/PG vesicles correlates with the antibacterial activity of the peptides against E. coli (Table II). [D]-L3,4,8,10-K5L7, which has the lowest antibacterial activity, also had significantly decreased ability to permeate PE/PG vesicles compared with the other three peptides.

Electron Microscopy Study of Bacterial Lysis—The effect of the diastereomers on the morphology of treated E. coli was visualized using transmission electron microscopy. All the peptides caused total lysis of the bacteria at the MIC (data not shown). However, when the peptides were utilized at concentrations corresponding to 80% of their MIC, some differences in the morphology of the treated bacteria were observed, depending upon the peptide used. The most hydrophobic peptide, [D]-L3,4,8,10-K5L7, caused the most damage to the cell wall and membranes, whereas the least hydrophobic peptide, [D]-L3,4,8,10-K5L7, only caused local perturbations (Fig. 3).

### DISCUSSION

Previous studies with model peptides used to elucidate the structure-function study of antibacterial peptides focused on three parameters: helical structure, hydrophobicity, and charge (20–24). Each change in one of these parameters simultaneously resulted in changes in the other two, making it difficult to clarify the unique contribution of each parameter to the overall antibacterial activity. In this study, the effect of the helical structure was eliminated, which therefore permitted the study of only two parameters, namely, hydrophobicity and net positive charge, by varying the ratio of leucine and lysine. For this purpose, we chose to investigate diastereomers of short model peptides (12 amino acids long) containing stretches of only 1–3 consecutive L-amino acids, which are too short to form an α-helical structure.

CD spectroscopy revealed that the diastereomers studied here are indeed totally devoid of α-helical structure (data not shown), unlike the diastereomers of melittin and pardaxin, which retain low α-helical structure (25, 26). Nevertheless, the diastereomers exhibit potent antibacterial activity similar to or greater than that of native antibacterial peptides such as dermaseptin S or the antibiotic drug tetracycline (Table II). Moreover, the most potent peptides, [D]-L3,4,8,10-K5L7 and [D]-L3,4,8,10-K5L7, were devoid of hemolytic activity against the highly cytolytically susceptible human erythrocytes. It should be noted that [D]-L3,4,8,10-K5L7, which is devoid of α-helical structure, has considerable hemolytic activity that approaches that of the native cytolytic peptide, pardaxin (13). This could indicate that the balance between hydrophobicity and positive charge compensates for the amphipathic α-helical structure. However, increasing the positive charge drastically reduced the hemolytic activity while antibacterial activity was preserved, demonstrating that α-helical structure is not required for antibacterial activity.

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**Table II**

| Peptide designation | Minimal inhibitory concentration of the peptides | E. coli (D21) | A. calcoaceticus (Ac11) | P. aeruginosa (ATCC-27853) | R. megestium (Bm11) | B. subtilis (ATCC-6051) |
|---------------------|-----------------------------------------------|---------------|-----------------|------------------|-----------------|------------------|
| [D]-L3,4,8,10-K5L7 | 9 | 20 | 125 | 0.7 | 1.1 | 1.1 |
| [D]-L3,4,8,10-K5L7 | 3.5 | 4 | 10 | 0.4 | 0.5 | 0.5 |
| [D]-L3,4,8,10-K5L7 | 7 | 20 | 10 | 0.25 | 2 | 2 |
| [D]-L3,4,8,10-K5L7 | 80 | 200 | >200 | 1 | 100 | 100 |
| Dermaseptin S | 6 | 3 | 25 | 0.5 | 4 | 4 |
| Melittin | 5 | 2 | 25 | 0.3 | 0.6 | 0.6 |
| Tetracycline | 1.5 | 1.5 | 50 | 1.2 | 6.5 | 6.5 |

* Results are the mean of three independent experiments, each performed in duplicates with a standard deviation of 20%.

**Table III**

| Peptide designation | Minimal inhibitory concentration | P. aeruginosa (ATCC-27853) | E. coli (D21) |
|---------------------|--------------------------------|-----------------|---------------|
| [D]-L3,4,8,10-K5L7 | 10 | 10 | 7 | 7 |
| Dermaseptin S | 25 | 200 | 6 | 50 |
| Tetracycline | 50 | 50 | 50 | 50 |
| Tetracycline + [D]-L3,4,8,10-K5L7 (1 μM) | 6 | 6 | 6 | 6 |

* Results are the mean of two independent experiments, each performed in duplicates, with a standard deviation of 20%.
The antibacterial peptide, magainin, is a nonhemolytic peptide found in the skin of Xenopus laevis. Magainin is a cationic, amphipathic peptide that has the ability to disrupt the membranes of Gram-positive and Gram-negative bacteria. The antibacterial activity of magainin appears to be due to its ability to insert into the bacterial membrane and form pores, leading to the lysis of the bacterium.

Magainin is composed of 25 amino acids and has a molecular weight of 2.7 kDa. It is a cationic peptide with a net charge of +12 at pH 7.0. Magainin has a high hydrophobicity, which allows it to insert into the lipid bilayer of the bacterial membrane. The hydrophobic core of the lipid bilayer is composed of fatty acyl chains, which are highly hydrophobic. Magainin's hydrophobic region is composed of a hydrophobic core of 16 amino acids, which is flanked by hydrophilic residues.

The antibacterial activity of magainin is due to its ability to form pores in the bacterial membrane. When magainin inserts into the membrane, it forms a channel that allows water and small molecules to pass through. This results in the leakage of intracellular components and the death of the bacterium.

Magainin is a member of a family of antimicrobial peptides called defensins, which are found in the skin and mucous membranes of vertebrates. Defensins are cationic, amphipathic peptides that have a high hydrophobicity and a high positive charge. They are able to insert into the lipid bilayer of the bacterial membrane and form pores, leading to the lysis of the bacterium.

Magainin has a high selectivity for Gram-negative bacteria, which is due to the differences in the lipid composition of the bacterial membrane. Gram-negative bacteria have an outer membrane, which contains lipopolysaccharides (LPS) and lipoteichoic acids (LTA). These polar lipids are not present in the bacterial membrane of Gram-positive bacteria. The presence of LPS and LTA in the outer membrane of Gram-negative bacteria provides a barrier to the insertion of magainin into the bacterial membrane.

The antibacterial activity of magainin is dependent on its ability to form pores in the bacterial membrane. The insertion of magainin into the membrane is driven by the hydrophobic interactions between the hydrophobic core of the peptide and the hydrophobic core of the lipid bilayer. The positive charge of magainin also plays a role in its ability to insert into the bacterial membrane. The positive charge of magainin provides electrostatic attraction to the negatively charged lipid head groups of the bacterial membrane.

The antibacterial activity of magainin is also dependent on its ability to form pores in the bacterial membrane. The formation of pores is a result of the insertion of the hydrophobic core of the peptide into the hydrophobic core of the lipid bilayer. The formation of pores allows water and small molecules to pass through, leading to the lysis of the bacterium.

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δ-toxin, the antibacterial peptide alamethicin, cobra direct lytic factor, and pardaxin exert several histopathological effects on various cells due to pore formation and activation of the arachidonic acid cascade. However, pardaxin diastereomers do not exert these activities (25). In addition, many amphipathic α-helical peptides bind to calmodulin and elicit several cell responses, and even all D-amino acid α-helices, including melittin have similar activity (41). Diastereomers with disrupted α-helical structure are not expected to bind to calmodulin. 2) Local D-amino acid substitution would result in controlled clearance of the antibacterial peptides by proteolytic enzymes, as opposed to the total protection acquired by complete D-amino acids substitution (42). Total resistance of a lytic peptide to degradation is disadvantageous for therapeutic use. Furthermore, the antigenicity of short fragments containing D,L-amino acids is dramatically altered as compared with their wholly L- or D-amino acid parent molecules (43). 3) Total inhibition of bacterial growth induced by the diastereomers is associated with total lysis of the bacterial wall, as shown by electron microscopy (Fig. 3). Therefore, bacteria might not easily develop resistance to drugs that trigger such a destructive mechanism. 4) [D]-L3,4,8,10-K5L7 has the ability to perturb the cell wall of bacteria at concentrations lower than their MIC, as seen by electron microscopy (Fig. 3). The simultaneous administration of clinically used antibiotics, which have no activity due to their inability to penetrate the bacterial cell wall, together with [D]-L3,4,8,10-K5L7 may present a solution to this resistance mechanism of bacteria.

In summary, the results obtained with pardaxin, melittin, and the model peptide diastereomers indicate that neither a specific sequence, length, or position of D-amino acids are required for a polypeptide to have antibacterial activity. However, these factors seem to be more crucial for cytotoxicity toward mammalian cells. Our results indicate that only modulating the hydrophobicity and net positive charge of linear cytotoxic polypeptides is sufficient in the design of a repertoire of potent antibacterial diastereomeric polypeptides for the treatment of infectious diseases.

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