Diastereomers of Cytolysins, a Novel Class of Potent Antibacterial Peptides*

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An amphipathic \( \alpha \)-helical structure is considered to be a prerequisite for the lytic activity of most short linear cytolytic polypeptides that act on both mammalian cells and bacteria. This structure allows them also to exert diverse pathological and pharmacological effects, presumably by mimicking protein components that are involved in membrane-related events. In this study \( \alpha \)-amino acid-incorporated analogues (diastereomers) of the cytolytin pardaxin, which is active against mammalian cells and bacteria, were synthesized and structurally and functionally characterized. We demonstrate that the diastereomers do not retain the \( \alpha \)-helical structure, which in turn abolishes their cytotoxic effects on mammalian cells. However, they retain a high antibacterial activity, which is expressed in a complete lysis of the bacteria, as revealed by negative staining electron microscopy. The disruption of the \( \alpha \)-helical structure should prevent the diastereomer analogues from permeating the bacterial wall by forming transmembrane pores but rather by dissolving the membrane as a detergent. These findings open the way for a new strategy in developing a novel class of highly potent antibacterial polypeptides for the treatment of infectious diseases, due to the increasing resistance of bacteria to the available antibacterial drugs.

In addition to or complementary to the highly specific cell-mediated immune response, vertebrates and other organisms have a defense system made up of distinct groups of broad spectrum antibacterial peptides (1, 2). One major group includes short linear polypeptides (\( -40 \) amino acids and less), which have been isolated from diverse species such as insects, amphibians, and mammals (1, 2). The largest family includes those polypeptides that are positively charged and adopt an amphipathic \( \alpha \)-helical structure. Some are cytotoxic to both bacteria and mammalian cells while others are active against only one or the other. Although the precise mechanism of the antibacterial activity of polycationic amphipathic \( \alpha \)-helical peptides is not yet fully understood, accumulating data suggest that they destroy the energy metabolism of the target organism by increasing the permeability of energy-transducing mem-

branes (3–5). The novel finding that all \( \alpha \)-d-amino acid polypeptides, which form a left-handed \( \alpha \)-helix, retain the antibacterial activity of the native peptides suggests that a chiral center is not involved in the lytic process (6, 7). Therefore, the target of these toxins is believed to be the cell membrane. Because of their amphipathic structure, it has been suggested that these antibacterial peptides permeate the membrane by forming ion channels/pores via a “barrel-stave” mechanism (8, 9). According to this model transmembrane amphipathic \( \alpha \)-helices form bundles in which outwardly directed hydrophobic surfaces interact with the lipid constituents of the membrane, while inwardly facing hydrophilic surfaces produce a pore. Alternatively, the peptides bind parallel to the surface of the membrane, cover the surface of the membrane in a “carpet”-like manner, and dissolve it like a detergent (10–12).

Pardaxin, a 33-mer polypeptide, is an excitatory neurotoxin that has been purified from the Red Sea Moses sole Pardachirus marmoratus (13, 14) and from the Peacock sole of the western Pacific Pardachirus pavoninus (15). Pardaxin possesses a variety of biological activities depending upon its concentration (reviewed in Ref. 16), and recently was found to be endowed with potent antibacterial activity (17). Its biological roles have been attributed to its interference with the ionic transport of the osmoregulatory system in epithelium and to presynaptic activity (18, 19) (reviewed in Ref. 16). Pardaxin has a helix-hinge-helix structure; the N-helix includes residues 7–11 and the C-helix includes residues 14–26. The helices are separated by a proline residue situated at position 13 (20). This structural motif is found both in antibacterial peptides that can act specifically on bacteria (e.g. cecropin) and in cytotoxic peptides that can lyse a variety of cells (e.g. melittin).

Herein, functional and structural studies with \( \alpha \)-amino acid-incorporated analogues (diastereomers) of pardaxin reveal that the \( \alpha \)-helical structure, while important for cytotoxicity toward mammalian cells, is not a prerequisite for antibacterial activity as the diastereomers can lyse bacteria completely as revealed by negative staining electron microscopy. The results are discussed in terms of proposed mechanisms of antibacterial activity as well as the advantages of this novel class of antibacterial peptides as potential drugs in the treatment of infectious diseases.

MATERIALS AND METHODS

Peptide Synthesis and Purification—The peptides were synthesized by a solid phase method on (phenylacetamido)diphenylcarbamoylmethyl-amino acid resin (0.5 mEq) (21). The resin-bound peptides were then transamminated with 30% ethylenediamine in dimethylformamide for 3 days, followed by filtration of the resin, precipitation of the protected peptides with ether, and removal of the protecting groups with HF. The synthetic peptides were purified (>95% homogeneity) by reverse-phase high performance liquid chromatography on a C18 column using a linear gradient of 25–80% acetonitrile in 0.1% trifluoroacetic acid for 40 min and then subjected to amino acid analysis to confirm their composition.

Antibacterial Activity Assay—The antibacterial activity assay was performed in sterilized 96-well plates (Nunc F96 microtiter plates) in a final volume of 100 \( \mu l \) as follows. Fifty microliters of a suspension containing bacteria at 1 \( \times 10^6 \) colony-forming units/ml in culture medium (LB medium) was added to 50 \( \mu l \) of water containing the peptide in serial 2-fold dilutions in water. Inhibition of growth was determined by measuring the absorbance at 492 nm, using a Microplate autoreader.
**RESULTS**

To examine the role of the α-helical structure of a polycationic cytolytic in its cytotoxicity toward mammalian cells and bacteria, a series of pardaxin-derived peptides (see Table I) were synthesized and characterized for their structure, hemolytic activity on human red blood cells (hRBCs), antibacterial activity, permeates negatively charged phospholipids, and in PBS (35 mM phosphate buffer, 0.15 M NaCl, pH 7.0). As expected, a dramatic decrease in the α-helix content while all the D-amino acid incorporated analogues gave very low signals that could not be attributed to specific structures (data not shown).

**Hemolytic and Antibacterial Activity of the Peptides—**The peptides were then examined for their hemolytic activity toward the highly susceptible human erythrocytes and for their potential to inhibit the growth of different species of bacteria. In addition, the cytotoxic bee venom mellitin, the antibacterial peptide dermaseptin, and the antibiotic tetracycline were used as controls. Fig. 2 shows the dose-response curves of the hemolytic activity of the peptides. Table II gives the MIC of the peptides for a representative set of test bacteria, which includes two Gram-negative species, E. coli and Acinetobacter calcoaceticus, and two Gram-positive species, Bacillus megaterium and Bacillus subtilis. The data reveal that (i) D-amino acids introduced into TAp in vitro dramatically reduced its hemolytic activity, which correlates with the loss of α-helix content in the corresponding analogues. TAp in vitro with the highest α-helix content is the most potent, while TAp with the lowest α-helix content is practically devoid of hemolytic activity up to the maximum concentration tested (50 μM). (ii) Despite the dramatic decrease in the α-helix content and hemolytic activity of the diastereomeric analogues, they all retained most of the potent antibacterial activity of the parent peptide, which is comparable with that of known native non-hemolytic antibacterial peptides (2) (Fig. 2).

**Membrane Destabilization by the Peptides—** A common property of all of the α-helical, positively charged, naturally occurring antibacterial peptides studied so far is their ability to interact and permeate negatively charged phospholipids better than zwitterionic phospholipids. The relevance of these findings to their biological target membranes has been attributed to the fact that the surface of bacteria contains lipopolysaccharides (in Gram-negative bacteria) and polysaccharides (teichoic acids, in Gram-positive bacteria), both of which are acidic, while normal mammalian cells (i.e., erythrocytes) express the predominantly zwitterionic phospholipid PC on their outer leaflet. Herein we demonstrated that TApL18L19, the only diastereomer that is devoid of hemolytic activity but retains antibacterial activity, permeates negatively charged phospholipids significantly better than zwitterionic phospholipids (Fig. 3). As such it behaves similar to native antibacterial peptides, although it is devoid of α-helical structure. The lack of significant intermediate activities with TApL7 and TApL18L19 might be explained by the fact that they both have either the hydropho-

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**Table I** Sequences and designations of the peptides investigated

| Peptide designation | Sequence<sup>a</sup> |
|---------------------|----------------------|
| TAp<sup>b</sup>     | GFFALIPKIISSPLFTKLLLSALVSALSSSLGGQG-(NH2)<sub>2</sub> |
| [D]P<sup>7</sup>     | GFFALIPKIISSPLFTKLLLSALVSALSSSLGGQG-(NH2)<sub>2</sub> |
| [D]L<sub>18</sub>L<sub>19</sub> | GFFALIPKIISSPLFTKL LLSALVSALSSSLGGQG-(NH2)<sub>2</sub> |
| [D]P<sup>L</sup>L<sub>18</sub>L<sub>19</sub> | GFFALIPKIISSPLFTKL LLSALVSALSSSLGGQG-(NH2)<sub>2</sub> |
| Mellitin<sup>b</sup> | GIAGAVELRLLTTGLPA ASDIWK RXQQ-(NH2)<sub>2</sub> |

<sup>a</sup> Underlined and bold amino acids were substituted with their D-enantiomers. E-(NH2)<sub>2</sub> stands for glutamic acid in which the two –COOH groups were modified to two –CO–NH–CH2CH2–NH2 groups by transamination with ethylenediamine.

<sup>b</sup> Underlined sequences designate the N and C helices, respectively.

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The abbreviations used are: hRBC, human red blood cell; MIC, minimal inhibitory concentration; diS-C<sub>2</sub>-5, 3,3-dichloro-1,1-diphenyl-1,2-carcino- nine iodide; PBS, phosphate-buffered saline; PC, egg phosphatidylcholine; PS, phosphatidylserine; TFE, 2,2,2-trifluoroethanol.
bic N helix or the amphipathic C helix intact, which is sufficient to promote strong binding to both types of vesicles via hydrophobic interactions.

Visualization of Bacterial Lysis Using Electron Microscopy—The effect of the peptides on the morphology of intact and treated bacteria was visualized using negative staining electron microscopy. Fig. 4 shows the photographs obtained with the non-hemolytic analogue [D]P7L18L19 as an example. It was found that at the MIC the peptide lysed the bacteria completely, and only small fragments could be observed (Fig. 4C).

However, at concentrations lower than the MIC, patches were observed (Fig. 4B), which might indicate the initial step involved in the lytic process.

**DISCUSSION**

Numerous studies have led to the conclusion that a net positive charge and an amphipathic α-helical structure are prerequisites for the activity of most of the linear antibacterial peptides studied so far. We therefore used TApar (net charge, +5), which has various cytotoxic and histopathological effects (16), as a case study.

Herein we demonstrate that TApar has an α-helical structure and is endowed with high antibacterial activity on Gram-negative and Gram-positive bacteria and with hemolytic activity on human erythrocytes. However, D-amino acids incorporated into TApar dramatically reduced its α-helical structure (Fig. 1). This in turn reduced the hemolytic activity of the diastereomeric analogues, which indicates the importance of this structure in the cytotoxicity of the peptide to mammalian cells. However, the amphipathic α-helical structure seems not to be

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**TABLE II**

| Bacterial species          | Strain | TApar | [D]P7 | [D]L18L19 | [D]P7L18L19 | Melittin | DermaseptinS | Tetracycline |
|----------------------------|--------|-------|-------|-----------|------------|----------|--------------|-------------|
| Escherichia coli           | D21    | 3     | 10    | 3.5       | 6          | 5        | 6            | 1.5         |
| Acinetobacter calcoaceticus| Ac11   | 3     | 5     | 2.5       | 6          | 2        | 3            | 1.5         |
| Bacillus megatetium        | Bm11   | 0.8   | 1.2   | 0.6       | 0.9        | 0.3      | 0.5          | 1.2         |
| Bacillus subtilis          | ATCC-6051 | 1.5  | 2     | 1.5       | 3          | 0.6      | 4            | 6.5         |
Peptides Derived from Diastereomers of Cytolysins

The activity of the peptides when there was a reduction of the tested substrate was no significant decrease in the antibacterial activity, since with most of the bacteria this is crucial for antibacterial activity, with the exception of Staphylococcus aureus. The structure of the antibacterial peptides by proteolytic enzymes rather than the total protection acquired by complete d-amino substitution (6). Total resistance of a lytic peptide to degradation might be a disadvantage in therapeutic use. Furthermore, short fragments containing d and L amino acids have a dramatically altered antigenicity as compared with their entire L- or d-amino acid parent molecules (31). (iii) It is evident from the electron micrographs that total inhibition of bacterial growth is associated with total lysis of the bacterial wall. Therefore, it might be more difficult for the bacteria to develop resistance to such a destructive mechanism, as compared with the more specific mechanisms of the commonly used drugs.

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