Peptidomimetic Antibiotics Target Outer-Membrane Biogenesis in Pseudomonas aeruginosa

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Antibiotics with new mechanisms of action are urgently required to combat the growing health threat posed by resistant pathogenic microorganisms. We synthesized a family of peptidomimetic antibiotics based on the antimicrobial peptide protegrin I (PG-I). These mimetics contain loop sequences related to that in PG-I, but linked to a D-proline–hairpin template, which helps to stabilize β-hairpin conformations within the macrocycle (4, 5) (Fig. 1A). One sequence variant, L2-1, had broad-spectrum antimicrobial activity like that of PG-I, but with a reduced hemolytic activity on human red blood cells (2). To optimize this lead, we performed iterative cycles of peptidomimetic library synthesis and screening for improved antimicrobial activity. The optimal hit from each library was used as a starting point for the synthesis and testing of variations in a subsequent library. This structure-activity trait led sequentially to mimetics L19-45, L26-19, and L27-11 (Fig. 1). L27-11

Naturally occurring peptides and proteins make interesting starting points for the design and synthesis of biologically active peptidomimetics. We previously synthesized libraries of β-hairpin–shaped peptidomimetics (1, 2) based on the membrane-lytic host-defense peptide protegrin I (PG-I) (3). These mimetics contain loop sequences related to that in PG-I, but linked to a D-proline–l-proline template, which helps to stabilize β-hairpin conformations within the macrocycle (4, 5) (Fig. 1A). One sequence variant, L8-1, had broad-spectrum antimicrobial activity like that of PG-I, but with a reduced hemolytic activity on human red blood cells (2). To optimize this lead, we performed iterative cycles of peptidomimetic library synthesis and screening for improved antimicrobial activity. The optimal hit from each library was used as a starting point for the synthesis and testing of variations in a subsequent library. This structure-activity trait led sequentially to mimetics L19-45, L26-19, and L27-11 (Fig. 1). L27-11

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possessed an interesting spectrum of antimicrobial activity, including minimal inhibitory concentrations (MICs) in the nanomolar range against many *Pseudomonas aeruginosa* (PA) strains and other *Pseudomonas* spp.; the mimetic is only weakly active or inactive against other Gram-positive and Gram-negative bacteria (Table I). In contrast, PG-1 displays broad-spectrum antimicrobial activity against Gram-positive and Gram-negative bacteria in the low micromolar range (7). The enantiomeric (mirror image) form of L27-11 is essentially inactive (MIC ≥ 32 μg/ml) against PA, unlike the two enantiomers of PG-1 (8), suggesting that the antibacterial target of the mimetic is chiral and not the achiral lipid chains of the cell membrane targeted by PG-1 (9–12). Further efforts focused on optimizing plasma stability and drug-like properties. The compounds POL7001 and POL7080 displayed much improved plasma half-lives while retaining a potent and selective action against PA; the MICs covering 90% of more than 100 PA clinical isolates tested (MIC₉₀) were 0.13 and 0.25 μg/ml, respectively, for these two mimetics. These isolates were of European and North American origin and most were resistant to one or more classes of clinically used antibiotics (6).

We next focused on identifying the mechanism of action of this family of peptidomimetics. Their mode of action against PA at concentrations close to the MIC was clearly different from the rapid membranolytic actions of both PG-I (7) and polymyxin B, a cationic macrocyclic peptide antibiotic of bacterial origin (13). Whereas PG-I or polymyxin at 4× MIC caused rapid cell lysis, the killing caused by the peptidomimetics was much slower (Fig. 1B). The ability of the peptidomimetics to permeabilize the PA cell membrane was also tested with the fluorescent nucleic acid stain SYTOX (14). No fluorescence increase was apparent over 120 min when PA cells were exposed to SYTOX and L26-19, L27-11, or POL7001, compared with a rapid fluorescence increase upon exposure to PG-I or polymyxin B (Fig. 1C (6)). A direct interaction between the peptidomimetic antibiotics and lipopolysaccharide (LPS) was examined with a dansyl-polymyxin displacement binding assay (15). This revealed an interaction between LPS and L27-11 in the low micromolar range [median inhibitory concentration (IC₅₀) 0.8 μM], which was not enantioselective (IC₅₀ 1.1 μM for the enantiomer of L27-11), suggesting that LPS is not the primary site of antibacterial action. Also, L26-19, L27-11, and POL7001 caused no notable lysis of human red blood cells at concentrations up to 100 μg/ml.

We tested whether the peptidomimetics have any effect on protein or nucleic acid biosynthesis in PA by examining the kinetics of incorporation of radiolabeled precursors into macromolecules. No notable influence was detected (6), suggesting that inhibition of protein, DNA, or RNA biosynthesis are not the primary modes of action. Similar attempts to monitor effects on cell wall biosynthesis by radiolabeled N-acetylglucosamine were frustrated by low levels of incorporation of this precursor into cell wall biosynthetics.

As an alternative approach to investigate the mechanism of action, a forward genetic screen was established to characterize the genetic basis for resistance to the antibiotics. Spontaneous resistant mutants of PA PAO1 could be selected on the antibiotic POL7080 at 5× MIC, at an estimated frequency of ≤ 1 × 10⁻¹⁰. Three mutants (PAO1RES1–3) showed MICs toward POL7080 and L26-19 of >32 μg/ml, but were more sensitive toward POL7001 (MIC 8 μg/ml versus 0.06 μg/ml for wild-type PA PAO1). No other changes in growth rate were observed. Compared to wild-type PA PAO1, the mutants showed only minor changes in sensitivity toward several other antibiotics (Table 2). To identify the causative mutation(s), we constructed three plasmid libraries from restriction-digested fragments of PA PAO1RES1 genomic DNA and transformed wild-type PA PAO1 with selection for growth on agar.

![Fig. 1. (A) Structures of PG-1 and peptidomimetics. Dashed lines indicate disulfide bridges in PG-1. The single-letter code is used for amino acids (30), except as shown, and X is L-2,4-diaminobutyric acid, ⁴P= d-proline, and ⁵P is l-proline. PAL-1 contains l-photo-Pro in place of ⁵P. (B) The kinetics of changes in growth rate were observed. Compared to wild-type PA PAO1, the mutants showed only minor changes in sensitivity toward several other antibiotics (Table 2). To identify the causative mutation(s), we constructed three plasmid libraries from restriction-digested fragments of PA PAO1RES1 genomic DNA and transformed wild-type PA PAO1 with selection for growth on agar.

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containing carbenicillin and POL7080. Resistant clones were isolated from all three libraries. Plasmid DNA isolated from 12 clones contained a common 5.4-kb overlapping DNA fragment [nucleotides (nt) 652,070 to 657,452 in the genome sequence (16) of PA PAO1] containing two contiguous open reading frames identified as homologs of surA and osta [also called imp and more recently lptD (used below)] (17). The 5.4-kb DNA fragment conferred the resistant phenotype on PA PAO1, whereas a smaller fragment containing only surA had no effect on resistance. The surA gene possessed the expected wild-type nucleotide sequence, whereas the lptD homolog from PA PAO1RES1 (here called lptDI) contained a mutation, consisting of a single 18–base pair tandem duplication of nt 628 to 645, corresponding to a tandem duplication of residues 210 to 215 with the sequence LRDEGM (6), with no other changes in the coding or upstream promoter sequences. The lptDI gene appears to act as a dominant resistance marker in the wild-type PAO1 PAO1 background. This does not appear to be a gene dosage effect, because introduction of the wild-type allele on plasmid pVLT31 (18) does not influence antibiotic sensitivity for PA PAO1 or PAO1RES1. Moreover, neither wild-type PAO1 PAO1, containing plasmid-borne copies of lptDI, nor PAO1RES1, containing copies of lptD, has altered sensitivity to most of the other antibiotics tested (Table 2). This argues against a general change in the permeability of the outer membrane caused by this mutation.

LptD is an outer-membrane protein widely distributed in Gram-negative bacteria that functions in the assembly of LPS in the outer leaflet of the outer membrane (17, 19–21). LptD is an essential low-abundance outer-membrane protein in Escherichia coli (19), depletion of which causes stress in outer-membrane biogenesis (21, 22). LptD in PA PAO1 is predicted to contain a C-terminal β-barrel domain (approximately residues 300 to 924) embedded in the outer membrane and an N-terminal domain (approximately residues 34 to 300) (19), which may reside in the periplasm and/or partly plug the β-barrel (Fig. 2A). The β-barrel sequence is highly conserved in LptD homologs in Gram-negative bacteria (17), although the N-terminal domain is more variable in length, comprising about 300 residues in PAO1 but only about 180 residues in E. coli K12 (6). The size difference in the periplasmic domains of LptD in E. coli and PAO1, the location within this domain of the lptD1 mutation, and the key function of LptD in outer-membrane biogenesis suggest that LptD may be a primary target of the peptidomimetic antibiotics.

Photoaffinity labeling experiments (23) were performed to determine whether the antibiotics bind to LptD in intact cells. For this task, an analog (PAL-1) was produced that contains a t-4,4-diazarainylproline (t-photo-proline) in place of L-proline, as well as a biotin tag at position 1 (MIC of PAL-1 against PAO1 = 0.05 µg/ml) (Fig. 1A) (6). Photoaffinity labeling with PAL-1 consistently revealed a major photolabeled protein with an apparent mass by SDS–polyacylamide gel electrophoresis of ≈100 kD, close to that expected for PAO1 LptD (calculated molecular weight = 100,751) (Fig. 2B). When the photoaffinity labeling was repeated in the presence of a 100× excess of L27-11, the labeled band disappeared from the blot, demonstrating a competition between L27-11 and PAL-1 for binding to LptD.

The identity of the photolabeled protein was proven to be LptD (PA0595) by two-dimensional (2D) gels and in-gel protease digestion/LC–ESI–MS–MS (liquid chromatography–electrospray ionization–tandem mass spectrometry) analysis and by immunoblotting with polyclonal antibodies raised against a synthetic C-terminal peptide fragment of LptD (6). When the photolabeling experiment was repeated with the resistant PAO1RES1 mutant, photolabeling of LptD was not detected, indicating that the protein was no longer able to bind the antibiotic with comparable affinity.

If the function of LptD is impaired upon binding to the peptidomimetic, then some effect on outer-membrane structure and biogenesis should become apparent (19, 24). When grown in Mueller-Hinton (MH) broth for several hours in the presence of growth-inhibitory amounts of L27-11, PAO1 cells became more sensitive to detergents ( Triton X-100 and SDS) and to various antibiotics, including tetracycline and rifampicin (6). PAO1 cells grown in MH broth with L27-11 or POL7001 were also examined in thin sections by transmission electron microscopy (TEM). This revealed internal accumulation of membrane-like material in many apparently intact cells (Fig. 2C), by two different fixation methods, an effect not seen in cells grown without the antibiotic. Similar accumulations have been observed in E. coli cells depleted of LptD (21, 22) and in other bacteria exposed to antimicrobial peptides (25). Some cells (≈10%) grown in this way formed filamentous comprising multiple concatenated cells (Fig. 2D), suggesting an impairment in cell division, which was not observed with untreated PAO1 PAO1 cells or with L27-11–treated PAO1RES1 cells. Cells grown in the presence of growth-inhibitory amounts of L27-11 could be uniformly stained with the membrane dye 3,5-dipropylthiacarbocyanine [diSC3(5)] (Fig. 2E), whereas untreated PAO1 or L27-11–treated PAO1RES1 cells exposed to this dye did not fluoresce. Thus, the action of L27-11 during growth impairs the outer-membrane permeability barrier.

Table 2. Antibacterial activities (MICs in MH-II broth) of various antibiotics toward wild-type PAO1 PAO1 (PAO1) and resistant mutant (PAO1RES1), as well as toward bacterial cells containing plasmid-borne copies of wild-type lptD or the resistance gene (lptD1) isolated from PAO1RES1. The plasmid vector used for these experiments (pVLT31) has no effect on resistance to the antibiotics shown, when introduced into PAO1RES1 or PAO1RES1.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>MIC (µg/ml)</th>
<th>PAO1WT</th>
<th>PAO1RES1</th>
<th>PAO1WT</th>
<th>PAO1RES1</th>
<th>PAO1WT</th>
<th>PAO1RES1</th>
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<tr>
<td>POL7080</td>
<td>0.06</td>
<td>64</td>
<td>4</td>
<td>64</td>
<td>4</td>
<td>64</td>
<td>4</td>
</tr>
<tr>
<td>L26-19</td>
<td>0.1</td>
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<td>16</td>
<td>&gt;64</td>
<td>&gt;64</td>
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</tr>
<tr>
<td>Gentamicin</td>
<td>0.5</td>
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<td>0.5</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tobramycin</td>
<td>0.25</td>
<td>0.25</td>
<td>0.12</td>
<td>0.25</td>
<td>0.25</td>
<td></td>
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</tr>
<tr>
<td>Ciprofloxacin</td>
<td>0.5</td>
<td>0.06</td>
<td>1</td>
<td>0.12</td>
<td>0.12</td>
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<tr>
<td>Colistin</td>
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<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.25</td>
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Interference with the function of LptD may also allow entry of phospholipids into the outer leaflet of the outer membrane. In E. coli and Salmonella spp., this is known to activate the outer-membrane enzyme PagP, which modifies LPS by converting the hexa-acyl form of lipid A into the hepta-acyl form by transferring a palmitate group from outer-leaflet phospholipids to lipid A (26, 27). The same effect has been observed in lptD-depleted E. coli (21). PAO1 may also modify its lipid A by addition of a C16 fatty acid, to generate from the normal penta-acylated form (molar weight 1447) a hexa-acylated derivative (molecular weight 1686) (28). This palmitate-containing hexa-acylated form has been observed in lipid A from PAO1 clinical isolates from patients with cystic fibrosis (29), although it is normally absent from laboratory-adapted strains (such as PAO1) (28).

We first confirmed that the lipid A prepared from LPS isolated from PAO1 PAO1 grown normally in MH broth was indeed the expected pentacylated form {negative-mode matrix-assisted laser desorption/ionization (MALDI)–MS mass/charge ratio (m/z) 1446 [M–H]–}. However, when cells were grown in MH broth with L27-11, after 5 hours the only lipid A detectable by MALDI–MS was the hexa-acylated form (observed mass m/z 1684 [M–H]–) containing a palmitoyl residue (6). A further indication of a perturbed membrane structure came from fractionation of membrane extracts from cells by sucrose density ultracentrifugation. Fractions containing LPS and LptD appeared at higher density in the gradient when they came from L27-11–cultivated cells as compared to untreated cells (6), a result similar to that reported for lptD-depleted E. coli cells (19, 21).
A fluorescently labeled derivative Fluo-1 (Fig. 1A) of L27-11 showed an MIC against PA PAO1 of 0.1 μg/ml, suggesting that it might label potential antibiotic binding sites. PA PAO1 cells in MH broth were incubated with Fluo-1 (5 μg/ml) for 1 hour at 37°C, washed, and examined by fluorescence microscopy. The resulting fluorescence staining was not uniform over the cell surface, but rather appeared to be concentrated in spots (Fig. 2F), suggesting a localized binding site. A similar labeling was not observed when the PA PAO1^RES1 mutant was stained in this way.

The ability of the antibiotics to provide protection against a lethal PA infection in a whole animal was also tested. For this, the in vivo efficacy of POL701 and POL7080 was evaluated in a mouse sepsis model at doses of 10, 3, 1, 0.3, and 0.1 mg per kilogram of body weight given subcutaneously at 1 and 5 hours after bacterial inoculation with either PA ATCC 9027 or ATCC 27853. Both antibiotics demonstrated substantial activity against both strains with calculated median effective doses (ED50) values in the range 0.25 to 0.55 mg/kg, as compared to gentamicin (used as a positive control), which showed an ED50 of 3.1 and 2.9 mg/kg, respectively, against these strains.

The mechanism of action of the peptidomimetic antibiotics reported here likely includes perturbation of the critical LPS transport function of LptD. The in vivo activity reported here raises the prospect that this family of antibiotics may be useful in a clinical setting to combat nosocomial infections and lung infections in patients with cystic fibrosis, where multiple drug-resistant PA strains are a serious health problem.

References and Notes
6. Supporting material is available on Science Online.
30. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; F, Phe; G, Gly; I, Ile; K, Lys; L, Leu; R, Arg; T, Thr; V, Val; W, Trp; Y, Tyr.
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Supporting Online Material
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Materials and Methods
Figs. S1 to S11
Tables S1 to S7
References
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**Killing *Pseudomonas***

Gram-negative *Pseudomonas* bacteria are opportunistic pathogens, and drug-resistant strains present a serious health problem. Srinivas *et al.* (p. 1010) synthesized a family of peptidomimetic antibiotics that is active only against *Pseudomonas*. These antibiotics do not lyse the cell membrane, but instead target an essential outer membrane protein, LptD, which plays a role in the assembly of lipopolysaccharide in the outer cell membrane. Activity in a mouse infection model suggests that the antibiotics might have therapeutic potential. In addition, LptD is widely distributed in gram-negative bacteria and so its validation as a target has the potential to drive development of antibiotics with a broader spectrum of activity against gram-negative pathogens.