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Peptidomimetic Antibiotics Target Outer Membrane Biogenesis in *Pseudomonas aeruginosa*

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One sentence summary:

A synthesized antibiotic targets a protein involved in outer membrane biogenesis to selectively kill *Pseudomonas* pathogens.

Abstract

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. Several rounds of optimization gave a lead compound that was active in the nanomolar range against gram-negative *Pseudomonas* sp., but was largely inactive against other Gram-negative and Gram-positive bacteria. Biochemical and genetic studies showed the peptidomimetics had a non-membrane-lytic mechanism of action and identified a homologue of the ß-barrel protein LptD (Imp/OstA), which functions in outer membrane biogenesis, as a cellular target. The peptidomimetic showed potent antimicrobial activity in a mouse septicemia infection model. Drug-resistant strains of *Pseudomonas* are a serious health problem, so this family of antibiotics may have important therapeutic applications.

Naturally occurring peptides and proteins make interesting starting points for the design and synthesis of biologically active peptidomimetics. We previously synthesized libraries of ß-hairpin-shaped peptidomimetics (*1, 2*) based on the membranolytic 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 ß-hairpin conformations within the macrocycle (*4, 5*) (Fig. 1A). One sequence variant L8-1 had broad-spectrum antimicrobial activity like 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 trail led sequentially to mimetics L19-45, L26-19 and L27-11 (Fig. 1). L27-11 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* sp.; the mimetic is only weakly active or inactive against other Gram-positive and Gram-negative bacteria (Table 1 (*6*)). In contrast, PG-I 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 $\geq$ 32 μg/ml) against *PA*, unlike the two enantiomers of PG-I (*8*), suggesting that the antibacterial target of the mimetic is chiral and not the achiral lipid chains of the cell membrane targeted by PG-I (*9-12*). Further efforts focused on optimizing plasma stability and drug-like properties. The compounds POL7001 and POL7080 displayed much improved plasma half-lives whilst retaining a potent and selective action against *PA*; the MICs covering 90% of more than 100 *PA* clinical isolates tested (MIC_{90}) were 0.13 and 0.25 μg/ml, respectively, for these two mimetics.

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 4x 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 using the fluorescent nucleic acid stain SYTOX (*14*). No significant 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 using a dansyl-polymyxin displacement binding assay (*15*). This revealed an interaction between LPS and L27-11 in the low micromolar range (IC_{50} 0.8 μM), which was not enantioselective (IC_{50} 1.1 μM for the enantiomer of L27-11), suggesting that LPS is not the primary site of antimicrobial action. Also, L26-19, L27-11 and POL7001 caused no significant 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 radio-labeled precursors into macromolecules. No significant 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 using radio-labeled N-acetylglucosamine were frustrated by low levels of incorporation of this precursor into cell wall biopolymers.

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 5x MIC, at an estimated frequency of $\leq 1x10^{10}$. Three mutants ($PAO1^{RES1-3}$) showed MICs towards POL7080 and L26-19 of >32 μg/ml, but were more sensitive towards POL7001 (MIC 8 μg/ml, vs. 0.06 μg/ml for wild-type (wt) *PA* PAO1). No other changes in growth rate were observed. Compared to wt *PA* PAO1 the mutants showed only minor changes in sensitivity towards several other antibiotics (see Table 2). To identify the causative mutation(s), we constructed three plasmid libraries from restriction-digested fragments of *PA* $PAO1^{RES1}$ genomic DNA and transformed wt *PA* PAO1 with selection for growth on agar 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 (nt 652070-657452 in the genome sequence (*16*) of *PA* PAO1) containing two contiguous open reading frames identified as homologues 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. Importantly, the *surA* gene possessed the expected wt nucleotide (nt) sequence, whereas the *lptD* homologue from *PA* $PAO1^{RES1}$ (called here *lptD1*) contained a mutation, comprising a single 18 bp tandem duplication of nt 628-645, corresponding to a tandem duplication of residues 210-215 with the sequence LRDKGM (*6*), with no other changes in the coding or upstream promoter sequences. The *lptD1* gene appears to act as a dominant resistance marker in the wt *PA* PAO1 background. This does not appear to be a gene dosage effect, since introduction of the wt allele on plasmid pVLT31 (*18*) does not influence antibiotic sensitivity for *PA* PAO1 nor $PAO1^{RES1}$. Moreover, neither wt *PA* PAO1 containing plasmid-borne copies of *lptD1*, nor $PAO1^{RES1}$ containing copies of *lptD* have

altered sensitivity to most of the other antibiotics tested (Table 2). This argues against a general change in the permeability of the outer membrane (OM) caused by this mutation.

LptD is an OM protein widely distributed in Gram-negative bacteria that functions in the assembly of LPS in the outer leaflet of the OM (*17, 19-21*). LptD is an essential low abundance OM protein in *E. coli* (*19*), whose depletion causes stress in OM biogenesis (*21, 22*). LptD in *PA* PAO1 is predicted to contain a C-terminal ß-barrel domain (approx. res. 300-924) embedded in the outer membrane and an N-terminal domain (approx. res. 34-300) (*19*), which may reside in the periplasm and/or partly plug the ß-barrel (Fig. 2A). The ß-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 *PA* but only about 180 residues in *E. coli* K12 (*6*). The size difference in the periplasmic domains of LptD in *E. coli* and *PA*, the location within this domain of the *lptD1* mutation, and the key function of LptD in OM 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, an analogue (PAL-1) was produced containing a *L*-4,4-diazarinylproline (*L*-photo-proline) in place of *L*-proline, as well as a biotin tag at position-1 (MIC of PAL-1 against *PA* 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-PAGE of ≈100 kDa, close to that expected for *PA* LptD ($MW_{calc.}$= 100,751) (Fig. 2B). When the photoaffinity labeling was repeated in the presence of a 100x 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) using 2D gels and in-gel protease digestion/LC-ESI-MS-MS analysis, and immunoblotting using polyclonal

antibodies raised against a synthetic C-terminal peptide fragment of LptD (*6*). When the photolabeling experiment was repeated using the resistant PAO1^{RES1} mutant, photolabeling of LptD was not detected, indicating that the protein is 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 OM 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, *PA* cells became more sensitive to detergents (Triton X-100 and SDS) and to various antibiotics, including tetracycline and rifampicin (*6*). *PA* PAO1 cells grown in MH broth with L27-11 or POL7001 were also examined in thin sections by transmission electron microscopy (EM). This revealed internal accumulation of membrane-like material in many apparently intact cells (Fig. 2C), using 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 filaments comprising multiple concatenated cells (Fig. 2D), suggesting an impairment in cell-division, which was not observed with untreated *PA* PAO1 cells, nor with L27-11-treated PAO1^{RES1} cells. Cells grown in the presence of growth-inhibitory amounts of L27-11 could be uniformly stained with the membrane dye 3,5-dipropylthiacarbocyanine (diSC$_3$(5)) (Fig. 2E), whereas untreated *PA* PAO1 or L27-11-treated PAOI^{RES1} cells exposed to this dye did not fluoresce. Thus, the action of L27-11 during growth impairs the OM permeability barrier.

Interference with the function of LptD may also allow entry of phospholipids into the outer leaflet of the OM. In *E. coli* and *Salmonella* sp. this is known to activate the OM 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*). *PA* can also modify its lipid A by addition of a C16 fatty acid, to generate from the normal penta-acylated form (MW 1447), a hexa-acylated derivative (MW 1686) (*28*). This palmitate-containing hexa-acylated form has been observed in lipid A from *PA* clinical isolates from cystic fibrosis (CF) patients (*29*), while it is normally absent from laboratory-adapted strains (such as PAO1) (*28*).

We first confirmed that the lipid-A prepared from LPS isolated from *PA* PAO1 grown normally in MH broth is indeed the expected penta-acylated form (negative mode MALDI-MS *m/z* 1446 $[M-H]^-$). However, when cells were grown in MH broth with L27-11, after 5 h 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 h 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 POL7001 and POL7080 was evaluated in a murine septicemia model at doses of 10, 3, 1, 0.3 and 0.1 mg/kg given subcutaneously at 1 and 5 h after bacterial inoculation with either *PA* ATCC 9027 or ATCC 27853. Both antibiotics demonstrated significant activity against both strains with calculated median effective doses (ED_{50} values) in the range 0.25-0.55 mg/kg, as compared to gentamicin (used as a positive control), which showed an ED_{50} of 3.1 and 2.9 mg/kg, respectively, against these strains.

In summary, we report a family of potent peptidomimetic antibiotics against *PA* that interact with the OM protein LptD, and cause major disturbances to membrane structure and biogenesis. The mechanism of action 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 CF, where multiple drug resistant *PA* strains are a serious health problem.

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Supporting Online Material

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Materials and Methods

Figs. S1 to S11

Tables S1 to S7

Figures

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, except as shown and X = 2,4-diaminobutyric acid, ^{D}P= D-proline and ^{L}P= L-proline. PAL-1 contains L-photo-Pro in place of ^{L}P. (**B**) The kinetics of bacterial cell death at 37^{o}C is shown for *PA* PAO1 in MH broth after treatment with the antibiotics at 4x MIC, or control (no drug). The remaining colony forming units (CFU) are shown as a function of time after exposure to the antibiotic. (**C**) *PA* membrane integrity was measured by fluorescence spectroscopy. *PA* PAO1 cells in MH broth were treated with SYTOX and each of the antibiotics (added at time t=0) at a concentration of 5 μg/ml. The fluorescence change is shown versus time.

Fig. 2. The predicted ß-barrel domain of LptD (924 residues) is shown integrated in the OM (**A**). (**B**) Photoaffinity labeling of membrane proteins in *PA* PAO1. PAL-1 (1 μg/ml) was incubated with *PA* cells at 37^{o}C and irradiated with UV light. Membrane proteins were extracted, separated from cytoplasmic proteins by ultracentrifugation, and subjected to gel electrophoresis. Biotinylated proteins were detected using a streptavidin-based chemiluminescence detection system after blotting to a PVDF-membrane; with (100x excess, lane B) and without (lane A) competing L27-11 during photolysis (*6*). (**C**)-(**F**) Effects on *PA* cells after growth for 1-3 h at 37^{o}C in MH broth either with L27-11 (+ antibiotic) or without antibiotic (no antibiotic); (**C**) transmission EM showing accumulation of extra membrane-like material within cryo-fixed *PA* PAO1 cells (bar = 0.1 μm); (**D**) formation of filaments comprising multiple concatenated *PA* PAO1 cells; (**E**) staining *PA* PAO1 with the membrane sensitive dye diSC$_3$(5) and washing. (**F**)

staining and light microscope images of wt *PA* PAO1 (*left*) and resistant mutant PAO1^{RES1} (*right*), after growth in the presence of Fluo-1 and washing.

Table 1. Antimicrobial activities of the peptidomimetics. The MIC values were determined by the microdilution method in MH broth in the presence of 0.02% bovine serum albumin (BSA). Note, the MIC values are typically 4-8 fold higher when the assays are performed in cation-adjusted MH (MH-II) broth in the presence of BSA. n.d. = not determined.

		MIC (μg/ml)					
Strain		L8-1	L19-45	L26-19	L27-11	POL7001	POL7080
P. aeruginosa	ATCC 27853	8	1	0.02	0.01	0.008	0.008
P. aeruginosa	PAO1	8	2	0.03	0.004	0.008	0.004
A. baumannii	DSM3008	n.d.	n.d.	>64	>64	>64	>64
K. pneumoniae	ATCC 13883	n.d.	n.d.	>64	>64	>64	>64
S. maltophilia	ATCC 13637	n.d.	n.d.	>64	>64	>64	>64
E. coli	ATCC 25922	8	8	32	64	>64	>64
E. faecalis	DSM 12956	n.d.	n.d.	>64	>64	>64	>64
S. aureus	ATCC 29213	8	64	64	>64	>64	>64

Table 2. Antibacterial activities (MICs in MH-II broth) of various antibiotics towards wt *PA* PAO1, resistant mutant (PAO1^{RES1}), as well as to bacterial cells containing plasmid-borne copies of wild type *lptD* or the resistance gene (*lptD1*) isolated from PAO1^{RES1}. The plasmid vector used for these experiments (pVLT31) has no effect on resistance to the antibiotics shown, when introduced into *PA* PAO1^{WT} or PAO1^{RES1}.

	MIC (μg/ml)				
clone / antibiotic	PAO1^{WT}	PAO1^{RES1}	PAO1^{WT} +*lptD1*	PAO1^{RES1} +*lptD*	PAO1^{RES1} +*lptD1*
POL7080	0.06	64	4	64	64
L26-19	0.1	>64	16	>64	>64
gentamicin	0.5	1	0.5	1	1
tobramycin	0.25	0.25	0.12	0.25	0.25
ciprofloxacin	0.5	0.06	1	0.12	0.12
colistin	1	0.5	0.5	0.5	0.25