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Mechanism of Uptake of Deglucoteicoplanin Amide Derivatives across Outer Membranes of *Escherichia coli* and *Pseudomonas aeruginosa*

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Teicoplanin is a glycopeptide antibiotic which is ineffective against gram-negative bacteria because of its inability to penetrate the outer membrane. Removal of the sugar residues and attachment of polyamines to carbon 63 yielded two dibasic deglucoteicoplanin amides, MDL 62,766 (766) and MDL 62,934 (934), with moderate MICs for *Escherichia coli* (2 to 4 μg/ml) and *Pseudomonas aeruginosa* (8 to 32 μg/ml) compared with those of the monobasic teicoplanin aglycone (16 and >1,024 μg/ml, respectively). MICs were increased 16- to 32-fold by Mg^{2+} supplementation of Luria broth but not by Na^{+} supplementation at an equivalent ionic strength. Both 766 and 934 were capable of binding to *P. aeruginosa* lipopolysaccharide (LPS) at Mg^{2+}-binding sites, as assessed by dansyl polymyxin displacement experiments. Furthermore, both compounds increased *E. coli* and *P. aeruginosa* outer membrane permeability to the hydrophobic fluorescent probe 1-*N*-phenylnaphthylamine (NPN), whereas the parent compounds teicoplanin aglycone and teicoplanin and the β-lactam ceftazidime were totally ineffective. Addition of 1 mM Mg^{2+} blocked the increase in outer membrane permeability. Compound 766 had a lower MIC than 934 for both bacteria tested, bound to LPS with a higher affinity, and permeabilized outer membranes to NPN at lower concentrations. We propose that both deglucoteicoplanin amides exhibit increased gram-negative activity by virtue of their ability to access the self-promoted uptake pathway across the outer membrane.

Glycopeptide antibiotics such as vancomycin and teicoplanin are high-molecular-weight inhibitors of peptidoglycan biosynthesis (4). They are characterized by having little or no activity against gram-negative bacteria (4), possibly because of their inability to penetrate the outer membrane, although they do have useful clinical activity against gram-positive bacteria, including streptococci and staphylococci. Vancomycin is a zwitterion, whereas teicoplanin has an extra negative charge. Recently, Gruppo Lepetit, an affiliate of Marion Merrell Dow, succeeded in producing the deglucoteicoplanin amides MDL 62,934 and MDL 62,766 (called here 934 and 766, respectively), which differ from teicoplanin by the removal of three sugar residues and the modification of a carboxyl residue shared by other glycopeptides to introduce a polyamine chain (Fig. 1). Thus these compounds have two positive and no negative charges. Interestingly, these modifications substantially enhanced the activity of the compounds against gram-negative bacteria. This situation, modification of a bulky antibiotic selective for a gram-positive bacterium by inclusion of a second positive charge to give rise to a compound with reasonable gram-negative activity, was directly analogous to that observed for azithromycin, a derivative of the macrolide erythromycin (2). Therefore, we hypothesized that the deglucoteicoplanin amides would, like azithromycin (2), have acquired increased activity against gram-negative bacteria by virtue of improved access to the self-promoted uptake pathway.

Hydrophilic antibiotics below a certain size exclusion limit cross the outer membranes of gram-negative bacteria through the channels of a class of proteins called porins (5, 6, 13). Both glycopeptide and macrolide antibiotics exceed this size exclusion limit and could not be expected to cross the outer membrane in this fashion, thus explaining the usual resistance of bacteria such as *Escherichia coli* and *Pseudomonas aeruginosa* to such antibiotics (6, 12, 13). In contrast, similar size limitations appear to be less important for nonporin pathways, including the hydrophobic uptake pathway and the self-promoted uptake pathway (12, 16). The former, however, is not of major importance in bacteria such as wild-type *E. coli* (12, 13) or *P. aeruginosa* (6) strains, which show substantial susceptibility to the deglucoteicoplanin amides 766 and/or 934, and thus was not considered here. Self-promoted uptake involves the interaction of cationic compounds with sites at the surface of the outer membrane at which divalent cations cross-bridge adjacent lipopolysaccharide (LPS) molecules (6). Because of their higher affinity for these sites, they displace these divalent cations, and, as a result of their bulky nature, cause distortion of the outer membrane and a consequent increase in outer membrane permeability. This increased permeability, manifested as increased uptake of probe molecules such as the hydrophobic fluorophor 1-*N*-phenylnaphthylamine (NPN), the protein lysozyme, and the β-lactam nitrocefin, is assumed to involve increased uptake of the interacting substance. Self-promoted uptake is directly relevant to subsequent bacterial killing, since mutants that demonstrate increased or decreased interaction of polycations with cell wall LPS are, respectively, supersusceptible (12) or resistant (2, 7, 15) to killing by these polycations. In this paper, we demonstrate data consistent with self-promoted uptake of the deglucoteicoplanin amides in both *E. coli* and *P. aeruginosa*.

MATERIALS AND METHODS

Strains and media. The standard strains utilized were *E. coli* K-12 strain UB1636 (*trp his lys rpsL lac lamB*/RP1) and

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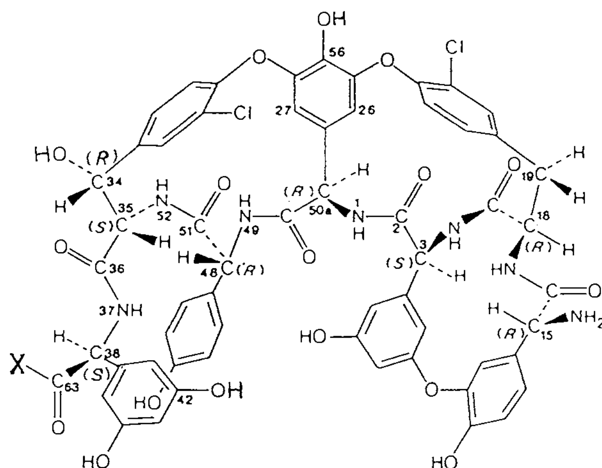

MDL	X	pI*	MW
62,766	$NH(CH_2)_3NH(CH_2)_3NH(CH_2)_3NH_2$	8.5	1369
62,934	$N(CH_3)(CH_2)_3NHCH_3$	8.1	1283
Teicoplanin aglycone	OH	5.5	1199

FIG. 1. Structures and chemical characteristics of the compounds 766 (MDL 62,766), 934 (MDL 62,934), and teicoplanin aglycone. MW, molecular weight. *, isoelectric point determined by isoelectric focusing.

P. aeruginosa PA01 strain H103, as previously described (7). Luria broth (LB) (1% Bacto Tryptone, 0.5% yeast extract; Difco Laboratories, Detroit, Mich.) was used for growth of *E. coli* and *P. aeruginosa*. NaCl (80 mM) and $MgCl_2$ (5 mM) were added as specified. MICs were determined by the broth microdilution method with an inoculum of 10^4 cells (7). Teicoplanin and derivatives were kindly provided by B. Goldstein, Marion Merrell Dow Research Institute, Gerenzano, Italy. They were previously published as teicoplanin aglycone (10), compound 20 (9) (called here 766), and compound 29 (9) (called here 934).

Permeabilization of outer membranes. A slight modification of the procedure of Hancock et al. (2, 7) was utilized. Cells were grown to an A_{600} of 0.5 and then centrifuged at 3,000 × *g* for 10 min at 23°C and resuspended at the same A_{600} in 5 mM sodium hydroxyethylpiperazine ethanesulfonate (HEPES) buffer (pH 7.2) containing 5 μM carbonyl cyanide *m*-chlorophenylhydrazone for *E. coli* or 1 mM sodium azide for *P. aeruginosa*. The cells were then washed again by centrifugation and resuspended at the same A_{600} in the same buffer solution. One milliliter of this bacterial suspension was placed in a glass cuvette and inserted into the cuvette holder of a Perkin-Elmer 650 10S spectrofluorometer. NPN from a 500 μM stock solution in acetone was added to a final concentration of 10 μM, and the background fluorescence due to binding of NPN to the cell surface, intrinsic bacterial fluorescence, and the intrinsic low-level fluorescence of NPN in aqueous solution at an excitation wavelength of 350 nm (slit width = 5 nm) and an emission wavelength of 420 nm (slit width = 5 nm) was recorded. The background fluorescence, which remained at a stable level for at least 10 min, was typically approximately 10 arbitrary units on the scale utilized in Fig. 2. Subsequently, a potential permeabilizing compound was added to the cuvette and the increased fluorescence emission was recorded as a function of time until a plateau was reached. Control experiments were performed to demonstrate that enhanced fluorescence was due to uptake of NPN into cells, as described previously (8). Briefly, cells were removed by centrifugation as described above and the residual NPN in the supernatant was determined by the fluorescence yield after addition of 3% Triton X-100. Experiments in which the effect of Mg^{2+} was measured were performed by preaddition of $MgCl_2$ to a final concentration of 1 mM prior to addition of the permeabilizing antibiotic.

Dansyl polymyxin displacement experiments. LPS was isolated from *P. aeruginosa* PAO1 strain H103 as previously described (11). Displacement of dansyl polymyxin from its cation-binding site on LPS was assessed as a decrease in dansyl polymyxin fluorescence as a function of added competitor, as previously described (11).

RESULTS

Teicoplanin had unmeasurably high MICs for both *E. coli* and *P. aeruginosa* (Table 1). This was in part because of the outer membrane permeability barrier in both bacteria, since the MICs for the barrier-defective mutants *E. coli* DC2 (1) and *P. aeruginosa* Z61 (7) were measurable, namely, 64 and 256 μg/ml, respectively. The parent compound of the deglucoteicoplanin amides (R = NH_2 in Fig. 1) had an MIC of 16 μg/ml for *E. coli* but an unmeasurable MIC for *P. aeruginosa* (Table 1) (9). In contrast, 766 had MICs of 2 μg/ml for *E. coli* and 8 μg/ml for *P. aeruginosa*, whereas 934 was two- to fourfold less effective for both species (Table 1) (9). The MIC was substantially medium dependent. For example, in min-

TABLE 1. Antibiotic MICs for *E. coli* and *P. aeruginosa* in the presence or absence of salt and Mg^{2+}

Organism	Additive	MIC (μg/ml)[a]						
		PX	GM	CTZ	766	934	TEIC	TEIC AG
E. coli	None	0.015	0.25	0.5	2	4	>1,024	16
	80 mM NaCl	0.015	0.25	0.5	2	4	>1,024	16
	5 mM $MgCl_2$	0.125	0.50	0.5	32	64	>1,024	32
P. aeruginosa	None	0.25	0.25	0.5	8	32	>1,024	>1,024
	80 mM NaCl	0.25	0.25	0.5	8	32	>1,024	>1,024
	5 mM $MgCl_2$	2	0.50	0.5	256	512	>1,024	>1,024

[a] Abbreviations: PX, polymyxin B; GM, gentamicin; CTZ, ceftazidime; TEIC, teicoplanin; TEIC AG, teicoplanin aglycone.

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TABLE 2. Kinetics of inhibition of binding of dansyl polymyxin to *P. aeruginosa* H103 LPS[a]

Compound	I_{50} (μM)	I_{max} (%)
Polymyxin B	3.3 ± 1.4	85 ± 6
Gentamicin	22 ± 20	47 ± 9
766	11 ± 5	63 ± 8
934	75 ± 24	34 ± 11
Teicoplanin	None[b]	None
Mg^{2+}	1,442 ± 919	67 ± 6

[a] All values are means ± standard deviations of 4 to 13 experiments using 0.25 μM LPS and 1.5 μM dansyl polymyxin.
[b] None, no inhibition.

imal medium with glucose as a carbon source, the MICs of 766 and 934 for *P. aeruginosa* were 1 and 2 μg/ml, respectively.

Antibiotics that are taken up by self-promoted uptake, such as polymyxin B, interact with the outer membrane at Mg^{2+} (divalent-cation)-binding sites (11). Thus Mg^{2+} is a competitive inhibitor of binding and, therefore, of activity of these antibiotics (7, 11) (Table 1). Consistent with this, Mg^{2+} increased by 16- to 32-fold the MICs of 766 and 934 for both *E. coli* and *P. aeruginosa*, whereas Na^+ at an equivalent ionic strength had no effect (Table 1). Similar data were obtained for a variety of other *E. coli* and *P. aeruginosa* strains (data not shown).

According to the self-promoted uptake hypothesis, the competition between Mg^{2+} and antibiotics taken up by this pathway is mediated by interaction with LPS (11, 16). To test this hypothesis, the ability of a variety of compounds to displace the probe molecule dansyl polymyxin from *P. aeruginosa* LPS was assessed. Competitive displacement experiments were performed at several concentrations, and the concentration leading to 50% maximal displacement (I_{50}) of dansyl polymyxin and the maximal displacement (I_{max}) of dansyl polymyxin were assessed. These data provided a measure of the relative affinities (I_{50}s) and relative numbers of binding sites (I_{max}s) of the compounds tested. Control experiments indicated that polymyxin B and gentamicin were approximately 500- and 65-fold more effective, respectively, at displacing dansyl polymyxin from LPS than was Mg^{2+}, which normally occupies these LPS binding sites. Similarly, 766 and 934 had I_{50} values 130- and 20-fold lower than that of Mg^{2+}, respectively, for dansyl polymyxin displacement, whereas teicoplanin failed to displace dansyl polymyxin from LPS. Consistent with the lesser effectiveness of 934 compared with that of 766, 934 had a sixfold-higher I_{50} (Table 2).

Similar to other compounds accessing self-promoted uptake, both 766 and 934 were effective in permeabilizing outer membranes of *E. coli* (Fig. 2A) and *P. aeruginosa* (Fig. 2B) to the hydrophobic fluorophor NPN. Control compounds polymyxin and gentamicin gave results similar to those observed previously, whereas ceftazidime (data not shown), teicoplanin, and the parent compound teicoplanin aglycone were ineffective at concentrations up to 200 μg/ml. Under the conditions used, there was no release into the supernatant of the TEM-1 β-lactamase expressed by plasmid RP1 in strain UB1636 at the concentrations of compound 766, polymyxin B, or gentamicin used in these studies, a result consistent with previous studies (6, 17). Thus these data did not reflect the creation of large holes in the outer membrane. Compound 766 demonstrated effectiveness similar to that of gentamicin in permeabilizing both *E. coli* (Fig. 2A) and *P. aeruginosa* (Fig. 2B) at low concentrations (around the MIC for 766). However, the maximal increase in NPN uptake was lower for 766. Consistent with its higher MICs and lower affinity for LPS, 934 demonstrated a substantially weaker ability to permeabilize both species (Fig. 2). Addition of 1 mM Mg^{2+} antagonized the promotion of NPN uptake by all tested compounds (data not shown).

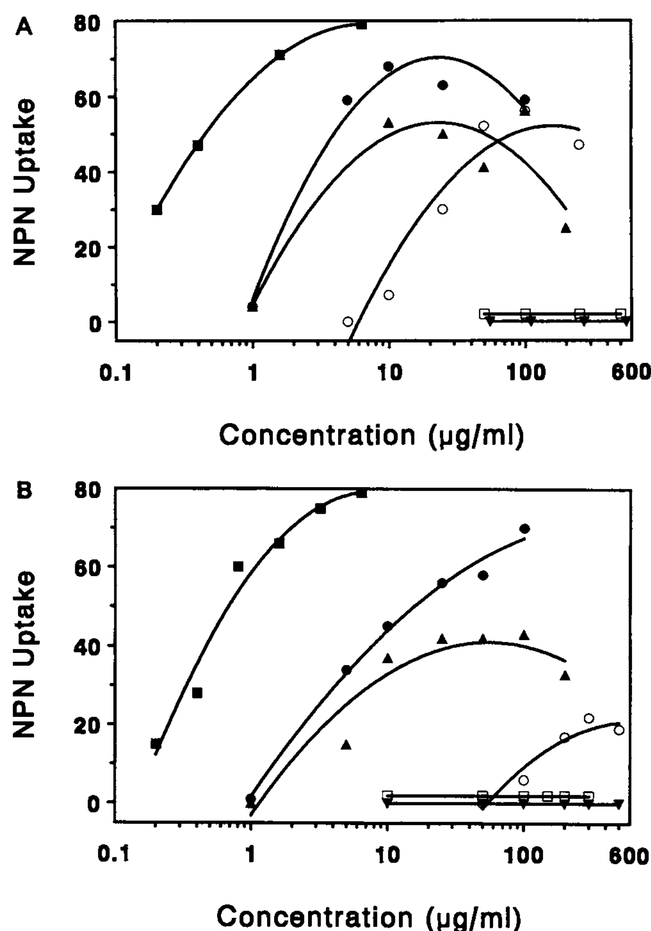

FIG. 2. Increase in outer membrane permeability towards the hydrophobic fluorescent probe NPN as a function of antibiotic concentration. NPN uptake, measured as fluorescence enhancement, is given in arbitrary units, with a maximal possible enhancement of 92 U. (A) *E. coli* UB1636. (B) *P. aeruginosa* H103. Symbols: filled squares, polymyxin; filled circles, gentamicin; upright triangles, 766; open circles, 934; open squares, teicoplanin aglycone; inverted triangles, teicoplanin.

DISCUSSION

The data presented in this paper are consistent with the conclusion that the novel deglucoteicoplanin amides are effective against *E. coli* and *P. aeruginosa* because of their uptake across the outer membrane via the self-promoted uptake route. Thus both 766 and 934 bound to the divalent-cation-binding sites of LPS with far higher affinities than the native divalent cations, promoted an increase in outer membrane permeability of the model compound NPN, and were antagonized by added excess Mg^{2+}. It should be noted that our MIC and Mg^{2+} antagonism studies were performed with Luria broth, which has a divalent-cation content of approx-

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imately 8.8 mg of Ca and 5.2 mg of Mg per liter (data provided by the manufacturer), i.e., virtually identical to that of Mueller-Hinton broth (cf. levels in serum of 45 to 98 mg of Ca and 13 to 22 mg of Mg per liter [3, 14]). Antagonism by 5 mM Mg^{2+} (120 mg/liter) was performed as an indicator of mechanism of uptake rather than in an attempt to predict therapeutic effectiveness. Taken together with results for the dibasic macrolide azithromycin (2), these data indicate a design principle that can be used to enhance the activity and spectrum of antibiotics that traditionally lack activity against gram-negative bacteria. Thus dibasic glycopeptide antibiotics such as 766 and 934 have MICs against *E. coli* and *P. aeruginosa* that are substantially lower than those of the parent compounds teicoplanin, which has two negative charges and one positive charge (4), and teicoplanin aglycone, with a single positive charge. These data correlate with the lack of ability of these parent compounds to increase the permeability to NPN of the outer membranes of *E. coli* and/or *P. aeruginosa*.

Polycationic compounds are commonly found in living cells and organisms. One example is the defensins, which are peptides found in the azurophilic granules of neutrophils. These peptides, which have molecular weights of more than 3,000 and four to nine positive charges, are also taken up by self-promoted uptake and are effective in both permeabilizing and killing cells at low molar concentrations (16). Thus it appears that while a high molecular weight can be a distinct disadvantage in porin-mediated uptake across the outer membrane, it appears to be of less importance in self-promoted uptake. Comparison of the MICs (Table 1) and NPN permeabilizing abilities (Fig. 2) for the various compounds taken up by self-promoted uptake reveals clear trends but no determinative relationship. In contrast, a relationship for related compounds such as the aminoglycosides (8) or the deglucoteicoplanin amides 766 and 934 and teicoplanin appears to exist. Thus, access to the self-promoted uptake pathway must depend in part on the molecular context of the positive charges that interact with the outer membrane. With this in mind, it should be possible to systematically design molecules which can optimally access the self-promoted uptake pathway.

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