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 Mg2+ 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 Mg2+-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 Mg2+ 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 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 divergent cations cross-bridge adjacent lipopolysaccharide (LPS) molecules (6). Because of their higher affinity for these sites, they displace these cationic 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
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 MgCl₂ (5 mM) were added as specified. MICs were determined by the broth microdilution method with an inoculum of 10⁴ cells (7). Teicoplanin and derivatives were kindly provided by B. Goldstein, Marion Merrell Dow Research Institute, Genzano, Italy. They were previously published as teicoplanin aglycone (10), compound 29 (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 A₆₀₀ of 0.5 and then centrifuged at 3,000 × g for 10 min at 23°C and resuspended at the same A₆₀₀ 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 A₆₀₀ 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 spectrophotometer. 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 Mg²⁺ was measured were performed by preaddition of MgCl₂ to a final concentration of 1 mM prior to addition of the permeabilizing antibiotic.

Dansyl polymyxin displacement experiments. LPS was isolated from P. aeruginosa PA01 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 261 (7) were measurable, namely, 64 and 256 μg/ml, respectively. The parent compound of the deglucotoicoplanin amides (R = NH₂ 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>
<thead>
<tr>
<th>Organism</th>
<th>Additive</th>
<th>PX</th>
<th>GM</th>
<th>CTZ</th>
<th>766</th>
<th>934</th>
<th>TEIC</th>
<th>TEIC AG</th>
</tr>
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<tbody>
<tr>
<td>E. coli</td>
<td>None</td>
<td>0.015</td>
<td>0.25</td>
<td>0.5</td>
<td>2</td>
<td>4</td>
<td>&gt;1,024</td>
<td>16</td>
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<tr>
<td></td>
<td>80 mM NaCl</td>
<td>0.015</td>
<td>0.25</td>
<td>0.5</td>
<td>2</td>
<td>4</td>
<td>&gt;1,024</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>5 mM MgCl₂</td>
<td>0.125</td>
<td>0.50</td>
<td>0.5</td>
<td>32</td>
<td>64</td>
<td>&gt;1,024</td>
<td>32</td>
</tr>
<tr>
<td>P. aeruginosa</td>
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<td>0.25</td>
<td>0.25</td>
<td>0.5</td>
<td>8</td>
<td>32</td>
<td>&gt;1,024</td>
<td>&gt;1,024</td>
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<tr>
<td></td>
<td>80 mM NaCl</td>
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<td>0.25</td>
<td>0.5</td>
<td>8</td>
<td>32</td>
<td>&gt;1,024</td>
<td>&gt;1,024</td>
</tr>
<tr>
<td></td>
<td>5 mM MgCl₂</td>
<td>2</td>
<td>0.50</td>
<td>0.5</td>
<td>256</td>
<td>512</td>
<td>&gt;1,024</td>
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</table>

* Abbreviations: PX, polymyxin B; GM, gentamicin; CTZ, ceftazidime; TEIC, teicoplanin; TEIC AG, teicoplanin aglycone.
Bacterial endotoxin (LPS) is a potent cytokine activator and an important component of the pathogenesis of Gram-negative infections. We have previously shown that the uptake of a hydrophobic fluorescent probe (NPN) is drastically increased by 100- to 1000-fold in membrane permeabilizing conditions, e.g., in the presence of divalent cations (c). Bellissent-Funel et al. have observed a similar effect using a variety of hydrophobic fluorophores and endotoxin preparations from different Gram-negative bacteria. The objective of this study was to analyze the uptake of NPN as a function of antibiotic concentration, and the effect of divalent cation in the uptake process.

The uptake of NPN into cell membrane preparations was monitored over a range of antibiotic concentrations. Antibiotics were added to the membrane suspension at various concentrations. The uptake of NPN was measured at different time points.

**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 Mg²⁺. It should be noted that our MIC and Mg²⁺ antagonism studies were performed with Luria broth, which has a divalent-cation content of approx-
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 Mg₂⁺ (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.

ACKNOWLEDGMENTS

Funding was provided through the Canadian Bacterial Diseases Network.

We acknowledge members of the Gruppo Lepetit, especially B. Goldstein, for providing us with these compounds, helpful discussion, and unpublished information, and A. Malabarba, who provided Fig. 1.

REFERENCES


