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Intracellular accumulation of linezolid in *Escherichia coli*, *Citrobacter freundii* and *Enterobacter aerogenes*: role of enhanced efflux pump activity and inactivation

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***Objectives*: The oxazolidinone class of antibiotics such as linezolid have a narrow spectrum of activity that targets Gram-positive bacteria. We hypothesized that the poor activity of linezolid in Gram-negative bacteria is in part caused by relatively low intracellular concentration due to efflux.**

***Methods*: Using whole cell accumulation assays we estimated the intracellular concentration of linezolid in *Escherichia coli* and other Enterobacteriaceae. We included test strains with enhanced RND-type multidrug efflux pump activity and with genetic inactivation of the pump or functional inhibition by carbonyl cyanide *m*-chlorophenylhydrazone as inhibitor of the proton motive force or 1-(1-naphthylmethyl)-piperazine (NMP), an efflux pump inhibitor.**

***Results*: Consistent with susceptibility studies, enhanced pump activity caused decreased accumulation, and pump inactivation and inhibition caused increased accumulation, of linezolid. The accumulation levels in test strains of *E. coli, Citrobacter freundii* and *Enterobacter aerogenes* with functional pumps were lower than in control strains of *Staphylococcus aureus* and *Enterococcus faecium*, but were higher after pump inactivation and correlated with ethidium bromide and pyronin Y accumulation.**

***Conclusions*: The intracellular concentration of linezolid is comparatively low owing to efficient efflux of the drug and could be increased substantially by inhibition of RND-type efflux pumps.**

Keywords: oxazolidinones, multidrug resistance, efflux pumps, Enterobacteriaceae

Introduction

Linezolid is the first member of the oxazolidinone group of compounds, a class of antibiotics that are chemically unrelated to other currently used antibacterial drugs. Oxazolidinones target the 50S subunit of the prokaryotic ribosome and thereby prevent assembly of the initiation complex, which is a different mode of action from that of other inhibitors of protein synthesis such as chloramphenicol and macrolides. Although the ribosomes of *Escherichia coli* are as susceptible to linezolid as those of Gram-positive cocci, linezolid MICs for Gram-negative bacteria are higher than those for Gram-positive cocci and usually lie above the breakpoint for resistance.[1] The activity of efflux pumps in Gram-negative bacteria appears to account for this finding: *E. coli* with inactivation of AcrAB, probably the most important RND (resistance–nodulation–cell division)-type multicomponent drug efflux pump in this species, is more susceptible to linezolid than cells with an intact AcrAB pump.[2,3] A small number of non-lytic arylpiperidines have been shown to increase the accumulation of radiolabelled linezolid in wild-type *E. coli* cells, whereas such an effect was not seen with the putative efflux pump inhibitor phenylalanine-arginyl-β-naphthylamide (PAβN) at

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a concentration that was effective in *Pseudomonas aeruginosa*.[4] We recently demonstrated that 1-(1-naphthylmethyl)-piperazine (NMP), another putative efflux pump inhibitor, reduced the MIC of linezolid by ≥4-fold for *E. coli, Citrobacter freundii, Enterobacter aerogenes* and *Acinetobacter baumannii*.[3,5–7] Although these findings suggest a role for RND-type efflux pumps in the susceptibility of Gram-negative bacteria to linezolid, confirmatory data showing corresponding differences in intracellular linezolid accumulation between Enterobacteriaceae with and without functional RND pump(s) and Gram-positive cocci are not available. In the present work, we used a specific HPLC assay to measure the intracellular linezolid concentration in *E. coli, E. aerogenes* and *C. freundii* strains with different efflux pump activity. The effects of NMP on the intracellular concentration of this drug were also assessed.

Materials and methods

Bacterial strains

E. coli K-12 strains AG100, 3-AG100, 3-AG100MKX, 1-DC14PS and 2-DC14PS as well as *E. aerogenes* strains EAEP289 and EAEP298 have previously been described (Table 1).[6,8–11] Reference strains of *C. freundii* (ATCC8090) and *E. aerogenes* (ATCC13048) were used in mutant selection experiments. After overnight incubation at 37°C in Luria–Bertani broth, cells were harvested and inocula of $\sim 10^{10}$ cfu were plated on LB agar containing inhibitory concentrations (2–16 times the MIC) of levofloxacin. Plates were incubated for 24 h at 37°C. Three to six single colonies from each selecting concentration, if available, were purified on fluoroquinolone-supplemented agar plates and examined for antimicrobial susceptibility. We obtained *C. freundii* mutant 2-CF and *E. aerogenes* mutant 1-EA that showed a multidrug-resistant (MDR) phenotype not seen in the parental strains. 2-CF overexpressed *acrB*; 1-EA had an undefined acquired mechanism of MDR, presumably associated with increased efflux pump activity. We constructed CFASII0 by means of inactivation of *acrB* in 2-CF, using the λ-based Red/ET homologous recombination system (Gene Bridges, Dresden, Germany).[12]

Susceptibility testing

Susceptibility to linezolid and ethidium bromide (EtBr) with or without NMP (final concentration, 100 mg/L) was studied by microbroth dilution with an inoculum of $\sim 5 \times 10^5$ cfu/mL and overnight incubation (~18 h) at 37°C in LB broth.

Table 1. Bacterial strains used in this study, with MICs of ethidium bromide (EtBr) and linezolid alone and after addition of NMP (100 mg/L); the results of pump gene expression studies [relative *acrB* or *acrF* (strain 2-DC14PS only) expression measured by quantitative RT–PCR, normalized for *gapA* expression] for selected strains are also shown

Species	Strain	Description	qRT–PCR of *acrB* or *acrF*	EtBr MIC (mg/L)		Linezolid MIC (mg/L)		Source or reference
				alone	+NMP	alone	+NMP	
E. coli	AG100	wild-type *E. coli* K-12 *argE3 thi-1 rpsL xyl mtl* Δ*(gal-uvrB)supE44*	1	256	32	128	16	8
	3-AG100MKX	*acrAB*-overexpressing *gyrA* mutant derived from AG100MK (AG100 *marA*::Kan^r)	ND	256	32	512	16	9
	3-AG100	*marR gyrA* mutant derived from AG100 after multistep selection with ofloxacin	7.2	512	128	1024	32	9
	1-DC14PS	*gyrA* mutant derived from DC14 (AG100 ΔacrAB::Kan^r) after selection with ofloxacin	ND	8	4	8	8	10
	2-DC14PS	*acrEF*-overexpressing mutant derived from 1DC14PS after selection with ofloxacin	1675	512	32	512	16	10
C. freundii	CF	wild-type *C. freundii* ATCC 8090	1	256	64	256	16	ATCC
	2-CF	*acrAB*-overexpressing mutant derived from CF after selection with levofloxacin	7.4	512	64	1024	16	this study
	CFAS0	CF Δ*acrAB*	ND	4	4	8	8	6
	CFASII0	2-CF Δ*acrAB*	ND	2	2	4	4	this study
E. aerogenes	EA	wild-type *E. aerogenes* ATCC 13048	1	≥1024	256	256	32	ATCC
	1-EA	derived from EA after selection with ofloxacin	0.98	≥1024	256	1024	32	this study
	EAEP 289	Kan^s derivative of EA27 (MDR clinical isolate; Kan^r Amp^r Chl^r Nal^r Str^r Tet^r)	ND	≥1024	256	1024	16	11
	EAEP 298	EAEP289 tolC::Kan^r (pEP786 integration)	ND	32	16	8	8	11

Quantitative RT–PCR of acrB and acrF

Gene transcription studies were done as described previously with some modification.[7] Cells were grown to mid-logarithmic phase, and RNA was isolated using the RNeasy Kit (Qiagen, Hilden, Germany). RNA samples were treated with DNase (Qiagen) on the column, quantified and stored at –80°C. An amount of 1 μg total RNA was reverse transcribed into cDNA using the iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) with an incubation of 45 min at 42°C. cDNA was stored at –20°C. PCR was carried out with the LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics, Germany). Primers were designed with the webtool primer 3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The primer sequences were: *E. coli acrB*, GAA CAA CTG GCG AGC AAA CT; *E. coli acrF*, AGT CTG AAA CCG TGG GAA GA; *C. freundii acrB*, TTA TCC CAA TGG CGT TCT TC; and *E. aerogenes acrB*, TCG CGT GAA GAA AGT GTA CG.

The 10 μL final volume contained 2.4 μL of $MgCl_2$ (25 mM), 0.5 μM of each primer, 1 μL of cDNA extract and 1 μL of Lightcycler Mix including FastStart *Taq* DNA polymerase, reaction buffer, dNTP mix, SYBR Green I dye and 10 mM $MgCl_2$. The PCR profile was as follows: denaturation at 95°C for 10 min and 30–40 cycles of 10 s at 95°C, 5 s at 55°C and 10 s at 72°C. Fluorescence was detected at the end of the 72°C segment in the PCR step (single mode). Expression of the housekeeping gene *gapA* (glyceraldehyde-3-phosphate dehydrogenase, forward primer sequence, CCA GAA CTT TGT TGG AGT AAC C; reverse primer sequence, AGC TTT AGC AGC ACC GGT A), was used as a relative standard.[13] The results of at least two independent RNA extractions were interpreted using the Relative Expression Software Tool (REST).[14]

Whole cell drug accumulation assays

EtBr and pyronin Y accumulation was measured by spectrum fluorometry as described previously.[5,6] For the estimation of the intracellular concentration of linezolid, cells were grown to logarithmic phase in LB broth at 37°C, washed in 50 mM sodium phosphate buffer (pH 7.0) and resuspended in the same buffer containing 0.4% glucose to an OD_{600} of 1.6–1.7. After an incubation of 10 min at 37°C, linezolid was added to a final concentration of 20 mg/L. At timed intervals, 1 mL samples were removed and centrifuged through silicone oil. The pellet was resuspended in 0.1 M glycine hydrochloride (pH 3.0). After overnight incubation at room temperature, samples were centrifuged and the supernatant was frozen until measurement. The HPLC analysis was performed following the method of Ehrlich *et al*.[15] Column switching was not necessary. In case of interferences with the matrix, the peak identity was determined by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Experiments included measurements after addition of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (300 μM) or NMP (100 mg/L). For selected strains, the NMP concentration was doubled (200 mg/L). All tests were done in triplicate. *Staphylococcus aureus* ATCC 29123 and *Enterococcus faecium* ATCC 6057 were included as Gram-positive control strains in the linezolid accumulation assays.

Results and discussion

MICs of linezolid (4–8 mg/L) and EtBr (2–16 mg/L) for efflux-deficient strains *E. coli* 1-DC14PS, *E. aerogenes* EAEP298 and *C. freundii* CFAS0 and CFASII0 were much lower than for wild-type strains or for strains overexpressing an RND-type efflux pump (Table 1). When NMP was added to either of the two drugs, MICs decreased ≥4-fold only for efflux-competent strains (Table 1). MICs in the presence of NMP were in all cases slightly higher than expected from efflux-deficient control strains. Thus, the transport inhibition by NMP at the concentration used was submaximal.

Accumulation data for the two dyes EtBr and pyronin Y and for linezolid are shown in Figures 1 and 2. Maximal accumulation of the three substances was observed in the efflux-incompetent strains, and lower intracellular concentrations were measured in the other test strains. *E. coli* wild-type strain AG100 accumulated less of the three substrates than the two other species, but in the case of linezolid and EtBr this did not perfectly correlate with the MICs. However, the relationship between accumulation in efflux-competent versus efflux-incompetent cells correlated well with the MICs for the test strains of a given species (Figures 1 and 2).

Addition of NMP and CCCP increased the intracellular concentration of linezolid (Figure 1). The increases with NMP were seen only in efflux-competent strains and were smaller than with CCCP, which is consistent with a submaximal effect of NMP, as seen in the MIC reduction assays. Alternatively, CCCP may have inhibited other pumps that depend on the proton-motive force and are able to bind and pump out linezolid, but are not inhibited as well by NMP. This is supported by the observation of linezolid accumulation levels increasing slightly with CCCP, even in efflux-'incompetent' cells. This effect was largest in *Enterobacter*. It is unknown whether the recently described *eefABC* efflux system which accommodates macrolides[16] can explain this finding. In AG100 and 3-AG100 we used an NMP concentration of 200 mg/L, which resulted in a large increase (2-fold) in the linezolid accumulation (data not shown), but the levels were still slightly lower than those obtained after CCCP addition.

At an extracellular concentration of 20 mg/L the level of linezolid in the supernatant fluid after cell lysis ranged between 0.21 (*E. coli* 3-AG100 and 3-AG100MKX) and 0.35 mg/L (*C. freundii* CF). With the same assay conditions the intracellular linezolid concentration in the two Gram-positive control organisms was higher (0.41 mg/L in both *S. aureus* and *E. faecium*). Linezolid levels could be increased by pump inactivation or inhibition to >0.5 mg/L, which is beyond the levels probably

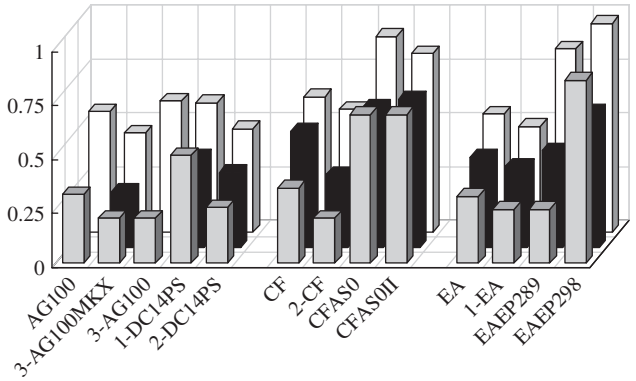

Figure 1. Whole cell accumulation of linezolid in *E. coli, C. freundii* and *E. aerogenes* with different levels of efflux pump activity. Linezolid (external concentration, 20 mg/L) was measured after 20 min (or 10 min after addition of NMP or CCCP) by an HPLC method. Shown are the means of triplicate experiments. Grey bars (first row) indicate accumulation without inhibitor, black bars (second row) represent accumulation levels in the presence of NMP and white bars (back row) represent accumulation levels in the presence of CCCP.

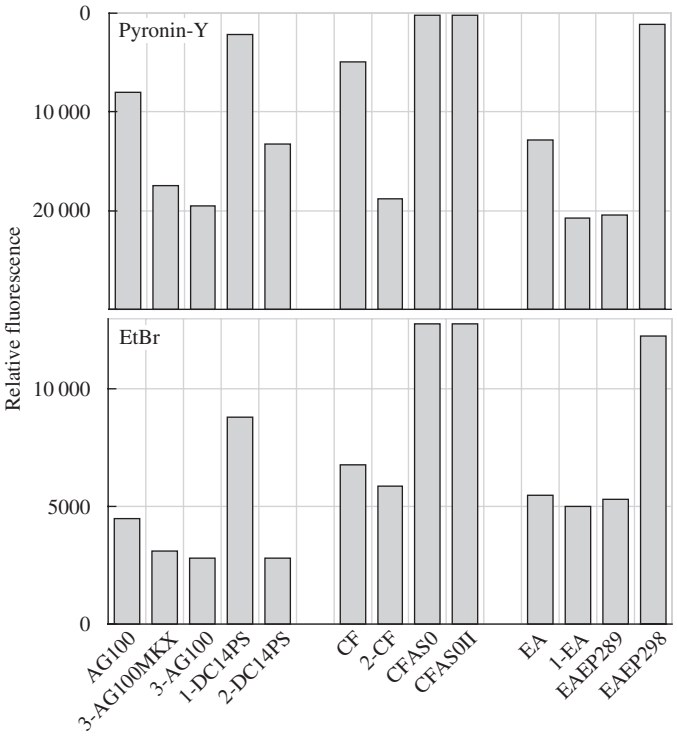

Figure 2. Whole cell accumulation of pyronin Y (upper panel) and EtBr (lower panel) in *E. coli, C. freundii* and *E. aerogenes* with different levels of efflux pump activity. Pyronin Y and EtBr were measured at 30 min by spectrum fluorimetry. The means of duplicate experiments are shown. Note that as intracellular pyronin Y accumulates and binds to RNA, the fluorescence is quenched; hence the fluorescence intensity inversely correlates with accumulation.

typical for *S. aureus* and enterococci although the MICs did not fall to a level <4–8 mg/L. Thus, the correlation between the intracellular accumulation of and susceptibility to linezolid was not perfect; MICs were 0.5–2 mg/L for Gram-positive control strains. Currently, there is no plausible explanation for this finding.

Linezolid, like macrolides and the recently described peptide deformylase inhibitors, has a narrow spectrum of activity, typically including Gram-positive pathogens. The observation that intracellular levels of linezolid could be increased by efflux inhibition to levels higher than those found in the typical target organisms is, we believe, noteworthy. With the increasing understanding of the mechanism of pump action and substrate binding sites it might be possible to design molecules that are less sensitive to efflux and/or that more efficiently inhibit relevant efflux pumps in Gram-negative bacteria.[17–19] Recycling of existing drugs, including narrow-spectrum drugs, for use in Gram-negative bacterial infections might then be a realistic goal.

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Transparency declarations

None to declare.

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