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Inhibitors of Antibiotic Efflux in Resistant *Enterobacter aerogenes* and *Klebsiella pneumoniae* Strains

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In *Enterobacter aerogenes* and *Klebsiella pneumoniae*, efflux provides efficient extrusion of antibiotics and contributes to the multidrug resistance phenotype. One of the alkoxyquinoline derivatives studied here, 2,8-dimethyl-4-(2′-pyrrolidinoethyl)-oxyquinoline, restores noticeable drug susceptibility to resistant clinical strains. Analyses of energy-dependent chloramphenicol efflux indicate that this compound inhibits the efflux pump mechanism and improves the activity of structurally unrelated antibiotics on multidrug-resistant *E. aerogenes* and *K. pneumoniae* isolates.

Various multidrug resistance (MDR) phenotypes that confer active protection against environmental toxic compounds by efflux mechanisms have been described in *Enterobacteriaceae* (1, 9, 16, 27, 28). One of these drug ejection systems, the efflux detected in resistant gram-negative bacteria, depends on membrane energy and efficiently expels structurally unrelated antibiotic molecules across the bacterial envelope via a tripartite complex comprising an inner membrane pump, a periplasmic fusion protein, and an outer membrane channel (26, 31).

Enterobacter aerogenes and *Klebsiella pneumoniae* are frequently described in resistant nosocomial infections (2–4, 10, 12, 24). In these bacteria, the *marRAB*, *acrAB-tolC*, and *ramA* genes are involved in expression of the MDR phenotype (8, 29, 32). Moreover, various clinical isolates show alteration of nonspecific porins associated with the presence of active drug efflux; both processes maintain a very low intracellular concentration of drugs and contribute to a high resistance level for structurally unrelated molecules including β-lactam antibiotics, quinolones, tetracyclines, and chloramphenicol (5, 6, 21, 24). An important medicinal challenge is to find new compounds capable of circumventing the efflux machinery (7, 19, 20, 22, 30). The aim of this study was to analyze 4-alkoxy-substituted quinolines, termed efflux pump inhibitors (EPI), with respect to their ability to interfere with the efflux pump.

The strains used in this work were *E. aerogenes* EA3, EA27, and EA117 and *K. pneumoniae* KP55 clinical isolates exhibiting active efflux of norfloxacin or chloramphenicol (6, 15, 21) and $TolC^-$ and $AcrA^-$ *E. aerogenes* EA27 derivatives previously constructed (29). MICs, chloramphenicol uptake, potassium efflux, and β-lactamase activities were determined as previously described (13, 21).

Biological effect of alkoxyquinolines on a resistant *E. aerogenes* strain. Documented clinical isolate EA27, overexpressing the AcrAB complex owing to a frameshift mutation in *acrR* (21, 29), was used to determine the activity of nine alkoxyquinolines. The alkoxyquinoline compounds and phenylalanine-arginine-β-naphthylamide (PAβN), a previously characterized EPI (20, 22), showed poor intrinsic antimicrobial activities with high MICs (Table 1). These low intrinsic activities allowed us to analyze the restoring effect of the molecules on the antibiotic susceptibility of several MDR strains. The various compounds were assayed for the ability to induce a decrease in the chloramphenicol resistance of *E. aerogenes* EA27 (Table 1). Compound 905 was effective as a reverse chemosensitizer of chloramphenicol susceptibility, with a 16-fold decrease in the MIC. This effect was observed at an alkoxyquinoline concentration corresponding to 1/10 of its proper MIC (Table 1).

The effects of compound 905 and PAβN on intracellular chloramphenicol accumulation were evaluated in strain EA27. Addition of compound 905 induced a twofold increase in the intracellular antibiotic concentration (Fig. 1), and we observed a similar accumulation in the presence of PAβN. These results suggest that compound 905 induces inhibition of chloramphenicol pump activity, the AcrAB/TolC system, which is overexpressed in this strain (29).

Effects of compound 905 and PAβN on *E. aerogenes* EA27 membrane. A major concern with the chemosensitizer is a possible permeabilizing effect on the membrane. To clarify this point, we analyzed membrane integrity in two independent ways. We measured potassium leakage (13) following addition of the alkoxyquinoline molecule. No significant K^+ release was observed after the addition of compound 905, even at a high concentration, whereas noticeable potassium release was obtained under the same conditions with polymyxin B, a well-known inducer of membrane permeabilization (data not shown). In addition, we tested the effects of compound 905 and PAβN on β-lactamase localization in EA27 cells. The products did not induce significant detection of periplasmic activity in the medium (Table 2), suggesting that compound 905 had no permeabilizing effect on *E. aerogenes* membrane under the conditions restoring antibiotic susceptibility.

Effect of compound 905 on the drug susceptibility of various efflux pump producers. We tested the activity of compound 905 in restoring susceptibility to structurally unrelated antibi-

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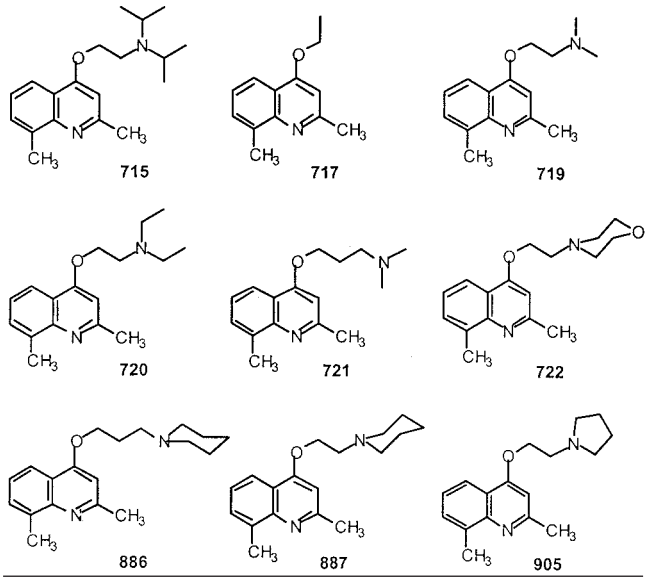
TABLE 1. Antibacterial and chemical properties of various alkoxyquinolines[a]



Inhibitor compound	MIC (mM)	Chloramphenicol MIC (μg/ml) in presence of inhibitor at:				
		0 mM	0.1 mM	0.2 mM	0.5 mM	1 mM
715	>10	512	512	512	512	256
717	2.5	512	512	512	256	256
719	5	512	512	512	256	128
720	5	512	512	512	256	128
721	5	512	512	512	256	128
722	10	512	512	512	512	256
886	5	512	512	512	256	256
887	10	512	512	512	512	512
905	>10	512	128/256	128	64	32
PAβN	1	512	64	64	32	ND[b]

[a] Syntheses of the compounds have been previously described (17). MICs were determinated in Mueller-Hinton broth as previously described on clinical isolate EA27 (21). The chloramphenicol susceptibility of *E. aerogenes* EA27 was tested in the presence of various concentrations of alkoxyquinolines or PAβN (0 to 1 mM). The underlined values correspond to chloramphenicol MICs obtained for a compound concentration corresponding to 1/10 of its proper MIC. Values are means of three independent determinations.

[b] ND, not determined (corresponding to the MIC of PAβN).

otic classes (Table 3). This compound increased the susceptibility of four clinical isolates, three resistant *E. aerogenes* strains and one resistant *K. pneumoniae* strain, for norfloxacin, tetracycline, and chloramphenicol (Table 3), which are efflux pump substrates (6, 15, 22). In contrast, we observed no significant variation in the MICs of cefepime. It is important to note that a severe alteration of porin, which is involved in the uptake of hydrophilic solutes, has been previously reported in the different isolates tested: EA3 synthesizes a channel-altered porin, while EA27, EA117, and KP55 produce very small porin amounts (6, 11, 15, 21). Mutations in the antibiotic target, e.g., substitutions in the QRDR domain of GyrA, have been reported in isolate EA117 (15).

To evaluate the contribution of compound 905 as a putative AcrAB/TolC pump inhibitor, we investigated the effect of compound 905 on EA27 and AcrA$^-$ and TolC$^-$ derivatives previously characterized (29). The chloramphenicol and norfloxacin

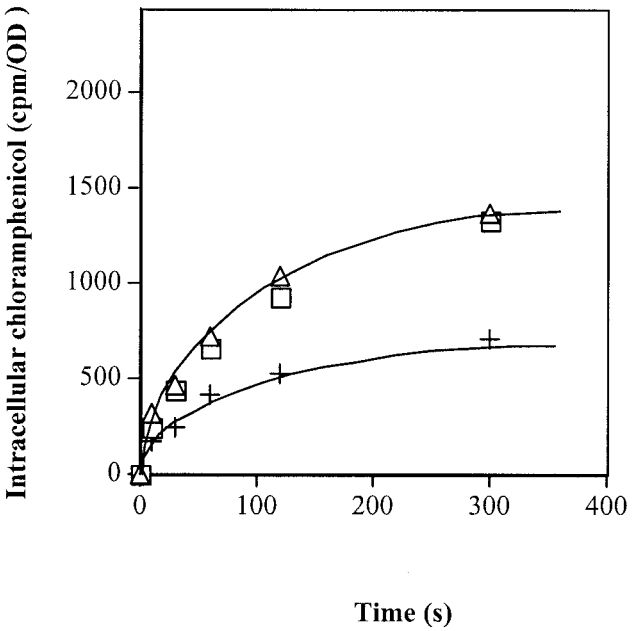


FIG. 1. Effect of compound 905 on chloramphenicol accumulation in *E. aerogenes* EA27. Exponential-phase bacteria in Luria-Bertani broth were removed, resuspended in sodium phosphate buffer, and incubated with radiolabeled chloramphenicol for various times (15, 22). Intracellular accumulations were followed in the absence (+) or presence of compound 905 (□) or PAβN (△) at 0.1 mM. Values (expressed as counts per minute/optical density [cpm/OD]) were obtained from independent duplicate measurements.

MICs for AcrA$^-$ and TolC$^-$ strains were not modified by the addition of compound 905 (Table 4). In addition, the AcrA or TolC knockout generated an increase in chloramphenicol and norfloxacin susceptibility (29) slightly greater than that obtained with the inhibitors in the parental strains (Table 4).

Identification of inhibitors of the efflux pump mechanism is of particular interest as regards the restoring intracellular antibiotic concentration. In this study, nine alkoxyquinolines were assayed on MDR clinical *E. aerogenes* strains as potential inhibitors of the efflux mechanism. Of these derivatives, one molecule, compound 905, induced an efficient increase in

TABLE 2. β-Lactamase localization and compound 905 or PAβN treatment[a]

Compound	β-Lactamase activity (mU) in:		β-Lactamase in medium (%)
	Cell suspension	Lysate of cell suspension	
None	24	640	3.8
905	30	670	4.5
PAβN	60	690	8.7

[a] The rate of nitrocefin hydrolysis was investigated in strain EA27 incubated in the absence or presence of PAβN or compound 905 (0.2 and 1 mM, respectively). Enzymatic activity was measured in equivalent amounts of intact cell suspension and the resulting total cell lysate obtained after sonication as previously described (21). The ratio of detected β-lactamase activity in cell suspension to that in lysate of cell suspension corresponds to the percentage of periplasmic activity detected in cell medium without cell disruption. One milliunit of β-lactamase was defined as the amount of enzyme that hydrolyzed 1 mmol of nitrocefin min^{-1} mg of protein^{-1} at 25°C (21). Values are means of two independent determinations.

TABLE 3. Effects of compound 905 on susceptibilities to structurally unrelated antibiotics in *E. aerogenes* EA3, EA27, and EA117 and *K. pneumoniae* KP55

Strain	Presence of compound 905 (1 mM)	MIC (μg/ml)[a]			
		CM	NFX	TC	CEF
EA27	−	512	256	16	32
EA27	+	32	64	4	64
EA3	−	512	128	8	64
EA3	+	32	32	0.5	ND
EA117	−	512	256	16	64
EA117	+	32	64	0.5	128
KP55	−	32	16	512	128
KP55	+	4	2	64	128

[a] Drugs were tested alone (−) or in the presence of alkoxyquinoline (+). MICs are means of three independent determinations. CM, chloramphenicol; NFX, norfloxacin; TC, tetracycline; CEF, cefepime; ND, not determined.

chloramphenicol, tetracycline, and fluoroquinolone susceptibility in various strains expressing the drug efflux process. The partial recovery of antibiotic susceptibility obtained with compound 905 is related to the other resistance mechanisms, mutation or modifying enzymes, reported in clinical isolates. Moreover, when compound 905 is added during incubation with chloramphenicol, an increase in intracellular antibiotic accumulation is observed in an MDR strain which overexpressed the AcrAB efflux pump (29). These results provide clear evidence that this alkoxyquinoline blocks antibiotic ejection in *E. aerogenes* and in *K. pneumoniae* clinical isolates. Although this response may be associated with an interfering effect that occurs during active pumping out of the antibiotic molecule, the efficiency of susceptibility restoration depends on the respective affinity of the transported drug and that of the competitor for the pump system involved in the efflux mechanism. A strong effect of compound 905 was observed on the drug susceptibility of strain EA27, which overexpresses the AcrAB pump, while no significant effect was obtained on the AcrA$^-$ and TolC$^-$ derivatives, for which the MICs of the corresponding antibiotics are lower.

Interestingly, we recently reported that 4-[2′-(piperidino) ethyl]-thioquinoline and 7-nitro-8-methyl-4-[2′-(piperidino) propyl]-thioquinoline at a high concentration are able to induce a slight increase in chloramphenicol susceptibility in strain EA27 (14). Similarly, 7-nitro-8-methyl-4-[2′-(piperidino) ethyl]-aminoquinoline blocks chloramphenicol efflux (23). Consequently, structure-activity relationship studies concerning homologous derivatives may be fruitfully undertaken to find more active molecules belonging to the series described here. Owing to the resolution of the three-dimensional structure of pump components (18, 25), the development of efficient responses to MDR bacterial pathogens with this family of inhibitors is open.

TABLE 4. Effects of compound 905 on the chloramphenicol and norfloxacin susceptibilities of EA27 and AcrA$^-$ and TolC$^-$ derivatives

Strain	Presence of inhibitor compound 905	MIC (μg/ml)[a]	
		CM	NFX
EA27	−	512	256
	+	32	64
TolC$^-$	−	32	16
	+	16	8
AcrA$^-$	−	32	32
	+	16	32

[a] Chloramphenicol (CM) and norfloxacin (NFX) were tested alone or in the presence of compound 905. MIC are means of three independent determinations.

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