Alkylaminoquinolines inhibit the bacterial antibiotic efflux pump in multidrug-resistant clinical isolates

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Over the last decade, MDR (multidrug resistance) has increased worldwide in microbial pathogens by efflux mechanisms, leading to treatment failures in human infections. Several Gram-negative bacteria efflux pumps have been described. These proteinaceous channels are capable of expelling structurally different drugs across the envelope and conferring antibiotic resistance in various bacterial pathogens. Combating antibiotic resistance is an urgency and the blocking of efflux pumps is an attractive response to the emergence of MDR phenotypes in infectious bacteria. In the present study, various alkylaminoquinolines were tested as potential inhibitors of drug transporters. We showed that alkylaminoquinolines are capable of restoring susceptibilities to structurally unrelated antibiotics in clinical isolates of MDR Gram-negative bacteria. Antibiotic efflux studies indicated that 7-nitro-8-methyl-4-[2′-(piperidino)ethyl]aminoquinoline acts as an inhibitor of the AcrAB–TolC efflux pump and restores a high level of intracellular drug concentration. Inhibitory activity of this alkylaminoquinoline is observed on clinical isolates showing different resistance phenotypes.

Key words: alkylaminoquinoline, antibiotic resistance, efflux inhibitor, efflux pump, multidrug resistance.

INTRODUCTION

Various MDR (multidrug resistance) systems have been characterized in Enterobacteriaceae [1–3]. Generally, they are well conserved and protect bacterial cells against environmentally toxic compounds [3–5]. The expression of such efflux systems is generally observed in clinical isolates and is associated with reduced drug uptake, owing to a decrease in the outer-membrane permeability [4,6,7]. The efflux mechanism detected in resistant Gram-negative bacteria provides efficient extrusion of antibiotic molecules across the membranes and periplasmic space using the membrane energy potential [4,6,7]. The efflux complex consists of three proteins, an inner-membrane pump, a periplasmic-membrane fusion protein and an outer-membrane channel [4,6,7].

The Gram-negative bacterium *Enterobacter aerogenes* is frequently responsible for nosocomial respiratory-tract infections [8–10]. We have recently identified the *marRAB* operon of *E. aerogenes* and characterized its role in controlling the regulation cascade involved in the expression of the MDR phenotype [11]. In various clinical isolates, a decreased synthesis of non-specific porins and the presence of an active drug efflux pump contribute to a high resistance level for structurally unrelated molecules, including β-lactam antibiotics, quinolones, tetracyclines and chloramphenicol [12–14]. Both mechanisms conjointly contribute to maintain the intracellular concentration of drugs below a toxic threshold [13]. In addition, this low internal amount is sufficient to de-repress the synthesis of deactivating enzymes conferring an additional resistance step [12,13].

Consequently, a new therapeutic challenge is to overcome this bacterial resistance strategy [15–17]. This can be achieved by either finding new bacterial targets or developing new molecules capable of circumventing resistance mechanisms. The purpose of the present study was to identify new molecules capable of restoring drug-susceptibility to different resistant isolates. The activity of various alkylaminoquinolines, as potential EPIs (efflux pump inhibitors), was analysed with respect to their effect on chloramphenicol efflux, and by comparison of the size and structure of their side chains. Two compounds exhibited very efficient EPI profiles and restored significant susceptibility for structurally unrelated antibiotics, such as norfloxacin, tetracycline and chloramphenicol, on *E. aerogenes* MDR clinical strains.

MATERIALS AND METHODS

Chemistry

The 4-alkylamino-substituted quinolines were prepared using synthetic pathways reported previously [18]. Crude samples were purified by crystallization. Purity was checked by TLC. Compounds were characterized by NMR spectrometry (^{1}H- and ^{13}C-NMR). Evaluation of side-chain bulk was achieved by calculating the van der Waals volume. For free rotating bonds, the final value taken into account results from the superimposition of all the possible staggered conformations including the most stable structure and those with energy no more 41 840 J/mol (10 kcal/mol), higher than that of stable conformer.

Bacterial strains, growth conditions and antibiotic susceptibility tests

E. aerogenes A.T.C.C. 13048 and previously described clinical isolates EA3, EA27 and EA117 were used [13,14,19–21]. Clinical strains EA3 and EA27 exhibit energy-dependent norfloxacin efflux [13]. Moreover, the AcrAB–TolC efflux pump is overexpressed in EA27 owing to an *acrR* mutation [22]. Bacteria were

Abbreviations used: EPI, efflux pump inhibitor; LB, Luria–Bertani; MDR, multidrug resistance; MIC, minimal inhibitory concentration; PAβN, phenylalanine arginine β-naphthylamide.

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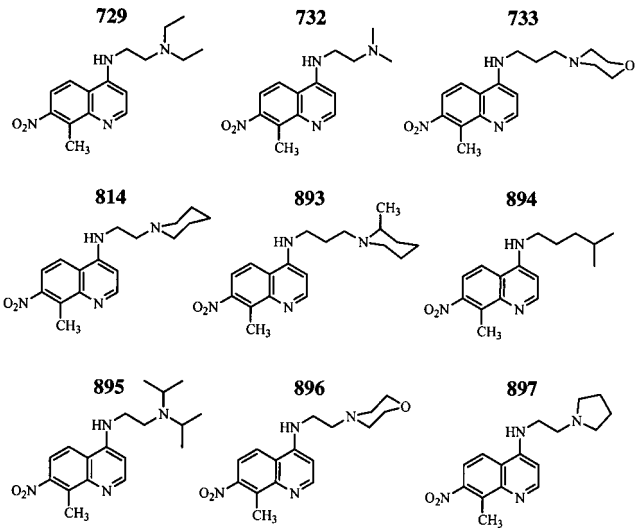

Figure 1 Chemical structure of the studied compounds

routinely grown in LB (Luria–Bertani) or Muller–Hinton broth at 37 °C. For the determination of MICs (minimal inhibitory concentrations), approx. 10^6 cells were inoculated into 1 ml of Muller–Hinton broth, containing 2-fold serial dilutions of each antibiotic. The results were read after 18 h at 37 °C [13,21]. To study the synergy activity, various concentrations of the potential inhibitors were added during the incubations with antibiotics [20,21].

Chloramphenicol uptake

The measurement of [^{14}C]chloramphenicol (Aventis Hoescht Marrion Roussel, Romainville, France) uptake by intact cells has been adapted from previous studies [13,21]. Briefly, exponential-phase bacteria grown in LB broth were pelleted, washed once and suspended to a density of 10^{10} c.f.u. (colony-forming units)/ml in 50 mM sodium phosphate buffer (pH 7), containing 5 mM magnesium chloride. [^{14}C]Chloramphenicol with a specific radioactivity of 59.46 mCi/mmol was added to 600 μl of cell suspension at 37 °C in a shaking water bath, which yielded a final chloramphenicol concentration of 5 μM. At various intervals, 100 μl of the suspension was removed and immediately filtered through GF/C filters (Whatman Ltd, Maidstone, Kent, U.K.). After three washes with 4 ml of cold phosphate/LiCl (0.1 M) buffer, filters were dried and the radioactivity was measured in a Packard scintillation counter. Inhibition assays were performed in the presence of alkylaminoquinoline 814 or PAβN (phenylalanine arginine β-naphthylamide) at the indicated final inhibitor concentration. Control samples were run under the same conditions.

RESULTS

Effects of alkylaminoquinoline drugs on *E. aerogenes*

E. aerogenes A.T.C.C. 13048 and EA27, a clinical isolate which exhibits an MDR phenotype [13,22], were used to determine the intrinsic biological activity of nine alkylaminoquinolines (Figure 1). As presented in Table 1, MICs of these compounds were high and similar to that obtained with PAβN, an EPI recently tested on *Pseudomonas aeruginosa* and on *E. aerogenes*-resistant strains [21,23,24]. Similar ranges of susceptibility values were obtained with the two strains tested. The low toxicity of these molecules (Table 1) allowed us to analyse their effect on the antibiotic susceptibility of the MDR strain. The nine molecules were assayed for their ability to decrease the level of chloramphenicol resistance to *E. aerogenes* EA27 (MIC = 512 μg/ml), which expresses an active efflux of chloramphenicol. Two compounds, 733 and 814, were particularly effective as potentiators of chloramphenicol activity *in vitro* with a 16-fold decrease in MIC observed at 0.5 and 0.2 mM concentration respectively (Table 2). This effect was observed at alkylaminoquinoline concentrations where no antibacterial activity was demonstrated on EA27. These results suggest that compounds 733 and 814 inhibit the chloramphenicol efflux pump.

Table 1 Antibacterial properties of alkylaminoquinolines on *E. aerogenes* strains

MICs were determined in Muller–Hinton broth as described previously [20,21] on A.T.C.C. 13048 and the EA27 clinical isolate. Values are means of three independent determinations.

		MIC (mM)	
Compounds	*E. aerogenes* strain . . .	A.T.C.C. 13048	EA27
729		1	0.5
732		0.5	0.5
733		1	1
814		0.5	1
893		1	1
894		0.25	0.5
895		1	1
896		1	2
897		0.5	0.5
PAβN		1	1

To compare the activity of the nine alkylaminoquinoline molecules, we determined the restoration of chloramphenicol susceptibility at the standard inhibitor concentration of 0.2 mM (Table 2). Compound 814 proved to be the most efficient inhibitor. It may be noted that a particular value of the side chain volume, approx. 135 Å^3 (1 Å = 0.1 nm), seems to be associated with the ability of the molecule to decrease the MIC of chloramphenicol (Table 2). This suggests that this volume may correspond to the minimal internal pocket diameter located in the channel of the efflux pump apparatus [25]. Alternatively, this result may support the hypothesis of a selective antibiotic-binding site, which could be masked by the inhibitor 814 and, consequently, inefficient to ensure the antibiotic transport.

Effect of alkylaminoquinoline 814 on chloramphenicol accumulation in *E. aerogenes* EA27

The effect of compound 814 on the intracellular accumulation of radiolabelled chloramphenicol was evaluated on the MDR strain EA27 for which active antibiotic efflux has been documented [13]. In this strain, the major efflux pump AcrAB–TolC is overproduced and generates a high drug-resistance level [22]. The presence of 814 significantly increased the intracellular chloramphenicol accumulation in EA27, and the level of accumulation induced by 814 corresponded to a 3-fold increase in intracellular antibiotic concentration (Figure 2). It is important to mention that the level of accumulated chloramphenicol observed in the presence of 814 was similar to that obtained in the presence of PAβN (Figure 2). These results demonstrate that 814 interferes with the

Table 2 Effect of alkylaminoquinolines on the chloramphenicol susceptibility of *E. aerogenes* EA27

Chloramphenicol was tested alone or in the presence of various alkylaminoquinoline molecules on the EA27 clinical isolate as described previously [13,20]. The minimal efficient concentration of the inhibitor is indicated. Values are means of three independent determinations. The MIC reduction factor was calculated from the chloramphenicol MIC value obtained in the absence or in the presence of alkylaminoquinolines (0.2 mM) on *E. aerogenes* EA27. Side-chain volumes were calculated with Sibyl (Tripos Inc., St Louis, MO, USA).

Compounds	Compound concentration (mM)	MIC (μg/ml) for chloramphenicol	Side chain volume ($\pm$5 Å^3)	MIC reduction (0.2 mM compound)
No compound	0	512	–	–
729	0.25	256	185	2
732	0.2	128	97	4
733	0.5	32	143	2
814	0.2	32	135	16
893	0.5	128	168	1
894	0.2	128	113	4
895	0.2	256	374	2
896	1	128	127	1
897	0.2	128	120	4
PAβN	0.2	64	–	8

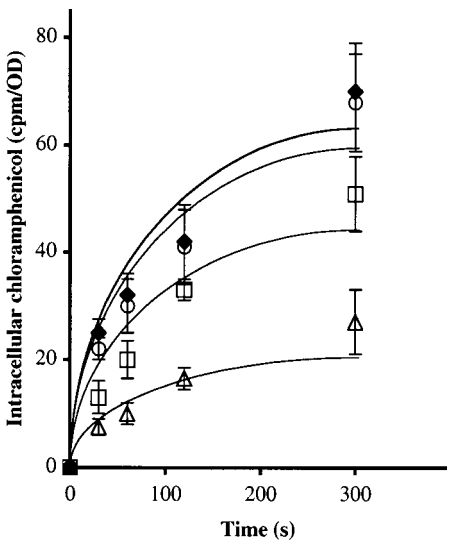

Figure 2 Effect of 814 on chloramphenicol accumulation in *E. aerogenes* EA27

Exponential-phase bacteria grown in LB broth were removed, resuspended in sodium phosphate buffer and incubated with radiolabelled chloramphenicol for various times. Intracellular accumulation was followed in the absence (△), in the presence of 0.2 mM PAβN (◆) or in the presence of 814 at the final concentration of 0.05 mM (□) or 0.1 mM (○). Values [expressed as c.p.m./*A* (OD)] were obtained from two independent experiments performed in duplicate.

Table 3 Effect of alkylaminoquinolines on susceptibilities of various *E. aerogenes* strains to structurally unrelated antibiotics

Drugs were tested alone (no compound), or in the presence of alkylaminoquinoline or PAβN at the indicated concentration of inhibitor on the A.T.C.C. 13048 sensitive strain and on various MDR clinical isolates as described previously [13,14,21,22]. Values are means from three independent determinations. Phenotypes of resistant clinical isolates are indicated: P+, normal level of porin expression; P↓, low level of porin expression; P°, expression of an altered-channel porin; P−, no porin expression; E−, no detectable efflux; E+, norfloxacin efflux; E‡, chloramphenicol efflux. CM, chloramphenicol; NFX, norfloxacin; TC, tetracycline; CEF, cefepime. n.d., not determined.

Compounds	Strain	Phenotype	MIC (μg/ml) for various antibiotics			
			CM	NFX	TC	CEF
No compound	A.T.C.C. 13048	P+, E−	8/4	0.5	2	0.5
	EA27	P−, E+	512	256	16	64
	EA117	P↓, E+, E‡	512	256	16	64
	EA3	P°, E+, E‡	512	256	8	64
733 (0.5 mM)	EA27	P−, E+	32/16	n.d.	4	32
	EA117	P↓, E+	32	64	4	32
814 (0.2 mM)	A.T.C.C. 13048	P+, E−	4	0.5	2	1
	EA27	P−, E+	32	32	4	64
	EA117	P↓, E+, E‡	32	32	0.5	32
	EA3	P °, E+, E‡	32	16	0.5	64
PAβN (0.1 mM) or (0.2 mM)*	A.T.C.C. 13048	P+, E−	4–4*	0.5–0.5*	2	0.5
	EA27	P−, E+	64–64*	128–128*	2	64
	EA117	P↓, E+, E‡	64	128	8	64
	EA3	P °, E+, E‡	64–64*	64	8	32

*, PAβN at 0.2 mM and the corresponding MIC results.

efflux activity, probably as a substrate competitor and, consequently, generates an increase in intracellular chloramphenicol concentration.

Alkylaminoquinolines and susceptibilities to structurally unrelated antibiotics in various *E. aerogenes* strains

It was important to assay the ability of 733 and 814 to restore the susceptibility towards structurally unrelated classes of antibiotics, which are also efflux pump substrates. To achieve this, products were tested on three clinical isolates concomitantly with norfloxacin, tetracycline, cefepime or chloramphenicol (Table 3). It must be noted that in the three clinical isolates tested, a severe decrease in porins involved in the uptake of hydrophilic solutes (decrease in porin synthesis or expression of a porin containing a hyper-constricted channel) has been reported previously in addition to the efflux mechanism: EA27 did not synthesize porins, whereas EA3 produced a channel-altered porin and EA117 produces less porin when compared with A.T.C.C. 13048 [13,14,19]. In addition, mutations in some antibiotic targets, e.g. substitutions in the quinolone-resistance-determining region of the gyrase [26] have been reported [13,20]. Interestingly, compounds 733 and 814 increased the susceptibilities to norfloxacin, tetracycline and chloramphenicol of all three MDR isolates for which an efflux process has been reported previously [13,14,21]. Moreover, both compounds induced a better recovery of antibiotic susceptibility than PAβN, especially for norfloxacin. The MICs for cefepime were not significantly modified, suggesting that cefepime is not a substrate of the efflux pump inhibited by the molecules tested. The major cefepime-resistance process is associated with the absence or alteration of the major unspecific

porin Omp36 [12,13,19]. We examined the effect of 814 and PAβN on the susceptible strain A.T.C.C. 13048. Results showed that these molecules were not active on a strain susceptible to various classes of antibiotics (Table 3). *E. aerogenes* isolates, namely EA27 and EA117, overproduce the AcrAB–TolC efflux pump, which confers a high level of resistance against various drugs [14,22]. Consequently, observations with the *E. aerogenes* MDR strains support the hypothesis that alkylaminoquinolines are efficient inhibitors of the efflux of various antibiotic molecules.

DISCUSSION

Combating antibiotic resistance is urgently needed and inhibition of efflux pumps is an attractive and powerful response to the emergence of MDR phenotypes in bacterial infections [27–29]. In this context, it is necessary to focus research on the targeting of these pumps to restore high intracellular concentration of structurally unrelated antibiotics and avoid inducing new mechanisms of resistance [15,16]. In the present study, quinoline derivatives were tested on *E. aerogenes* MDR clinical isolates as potential inhibitors of drug efflux pumps. The structure–activity relationships of these molecules were investigated by changing the side-chain group. It must be noted that only piperidinoethyl (814) and morpholinopropyl (733) branched side chains confer significant inhibitory activity against the assayed molecules. Compound 814 presented the highest biological activity at the minimal concentration. When 814 was used in combination with chloramphenicol, an increase in antibiotic intracellular accumulation was observed in an MDR strain expressing active drug efflux. In addition, this increase was dose-dependent. These results provide clear evidence that compound 814 inhibits antibiotic ejection. In *E. aerogenes*, AcrAB-TolC is the main efflux pump involved in the antibiotic resistance of clinical isolates [22]. The *Escherichia coli* TolC structure [30,31] shows that the channel internal diameter is approx. 35 Å, as the maximal hindrance diameter displayed by alkylaminoquinolines is approx. 20 Å, we can hypothesize that the pump inhibition takes place either on the inner-membrane transporter or at the inner-pump–outer-channel junction, which presents a restriction after the central cavity [25]. According to our results, the group branched in the side position of the quinoline heterocyclic moiety could play a strategic role in the recognition of or competition with the putative affinity sites required for antibiotic efflux via the AcrAB–TolC complex.

In addition, the difference in the level of inhibition conferred by PAβN compared with compound 814 observed in the present study may suggest that the inhibitory activity is related to the drug-binding domain [32], recently identified in the central cavity of an inner-membrane transporter [33–35]. This domain is located in the pore region of the AcrB headpiece [25]. Consequently, the efficiency of an inhibitor may reflect its affinity for the site located in the transporter, and AcrB was targeted during our screening procedure on *E. aerogenes*, whereas PAβN has been selected for its efficiency on *P. aeruginosa* efflux pumps [23]. Alternatively, the side chain of alkylaminoquinolines could generate steric hindrances in the pump channel, particularly in the restricted pore region [25] and thus impair antibiotic efflux. It is important to note that the most active molecule at the lowest concentration tested, e.g. 814, exhibits a side-chain volume close to 135 ± 5 Å^3, whereas the most stable three-dimensional ring geometry of compounds generated by conformational analysis is quite similar for all the molecules. These results suggest a role of this part of the molecule during the blocking of efflux mechanism.

Moreover, this compound restored susceptibility of *E. aerogenes* MDR clinical strains to structurally unrelated antibiotics such as norfloxacin, tetracycline and chloramphenicol. The magnitude of the 814 effect on drug susceptibility depends on the phenotype of isolate, which can also include mutation of the drug target, modifying enzymes and alteration of membrane permeability, in addition to the efflux mechanism. However, the benefit obtained with 814 is significantly better than that obtained with PAβN for the three structural antibiotic classes tested on *E. aerogenes*. This advantage could be attributed to differences in the internal structure of the active efflux pump in both *E. aerogenes* and *P. aeruginosa* [33,34]. PAβN, whose volume is more than that of 814, could encounter additional steric hindrances, which impair the effective access of the inhibitor to the site located inside the pump cavity of *E. aerogenes*.

In conclusion, this is the first description of molecules which efficiently inhibit efflux of various classes of antibiotics in *E. aerogenes*, an emerging MDR nosocomial pathogen. The inhibitory activity of alkylaminoquinolines is observed on clinical isolates showing different resistance phenotypes. With the recent structure resolution of the AcrB pump and the AcrB–drug complexes [25,32], these inhibitors offer the perspective to build specific molecules to investigate the structure–function relationships of drug transporters and develop efficient original weapons against MDR bacterial pathogens.

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