An alkylaminoquinazoline restores antibiotic activity in Gram-negative resistant isolates

Abdallah Mahamoud,[†] Jacqueline Chevalier,[†] Milad Baitiche,[‡]
Elissavet Adam and Jean-Marie Pagès

Correspondence
Jean-Marie Pagès
jean-marie.pages@univmed.fr

UMR-MD-1, Facultés de Médecine et de Pharmacie, Université de la Méditerranée, Marseille, France

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To date, various bacterial drug efflux pump inhibitors (EPIs) have been described. They exhibit variability in their activity spectrum with respect to antibiotic structural class and bacterial species. Among the various 4-alkylaminoquinazoline derivatives synthesized and studied in this work, one molecule, 1167, increased the susceptibility of important human-pathogenic, resistant, Gram-negative bacteria towards different antibiotic classes. This 4-(3-morpholinopropylamino)-quinazoline induced an increase in the activity of chloramphenicol, nalidixic acid, norfloxacin and sparfloxacin, which are substrates of the AcrAB-TolC and MexAB-OprM efflux pumps that act in these multidrug-resistant isolates. In addition, 1167 increased the intracellular concentration of chloramphenicol in efflux pump-overproducing strains. The rate of restoration depended on the structure of the antibiotic, suggesting that different sites in the efflux pumps may be involved. A molecule exhibiting a morpholine functional group and a propyl extension of the side chain was more active.

INTRODUCTION

The continual emergence of bacterial multidrug-resistant (MDR) strains is an increasingly important healthcare problem, and the dissemination of MDR pathogens severely compromises the efficacy of antibiotic treatment (Blot *et al.*, 2007; Chopra *et al.*, 2008; Falagas & Bliziotis, 2007). Bacterial efflux transporters pump all antibiotic classes out of the cell before they can reach their intended targets, and confer a noticeable decrease in susceptibility that favours the acquisition of specific resistance mechanisms (Davin-Régli *et al.*, 2008; Li & Nikaido, 2009; Piddock, 2006; Poole, 2007). The overexpression of the AcrAB-TolC pump belonging to the RND transporter family has been reported in several large collections of resistant clinical isolates (Davin-Régli *et al.*, 2008; Li & Nikaido, 2009; Nikaido & Takatsuka, 2009).

Similarly to the combination of a β -lactam and a β -lactamase inhibitor, e.g. amoxicillin plus clavulanate or piperacillin plus tazobactam, a new strategy has emerged for the development of efflux pump inhibitors (EPIs) to be used in combination with the expelled antibiotics (Lomovskaya & Bostian, 2006; Pagès & Amaral, 2009;

Pagès *et al.*, 2010). This new group of antibacterial molecules is not yet in clinical use, but several studies have demonstrated their *in vitro* ability to enhance the activity of antibiotics against efflux mediated by MDR bacteria (Lomovskaya & Bostian, 2006; Pagès & Amaral, 2009). Phenylalanine-arginine β -naphthylamide (PA β N), the pioneer molecule of this new antibacterial class, defined as an anti-resistance agent, has been extensively employed as an EPI reference in various studies (Lomovskaya & Bostian, 2006; Pagès & Amaral, 2009). Disadvantages of this molecule are its toxicity and low stability, which strongly limit its clinical potential. Consequently, the development of derivatives and the search for other compounds have been stimulated (Lomovskaya & Bostian, 2006; Pagès & Amaral, 2009).

Several compounds belonging to the quinoline family have been studied for their ability to restore the activity of antibiotics expelled by an efflux pump (Pagès & Amaral, 2009; Ghisalberti *et al.*, 2005). Some compounds are active towards MDR *Enterobacter aerogenes* clinical isolates that overexpress efflux pumps. Their activity has also been demonstrated by an increase in the intracellular concentrations of radiolabelled antibiotics that are substrates of the efflux pumps expressed in MDR isolates. This family of molecules may act as blockers of antibiotic transport within the AcrB pump (Pagès & Amaral, 2009). Recently, we have described the synthesis and characterization of a novel class of quinazoline compounds that exhibits the interesting ability to block the efflux pumps that act in resistant strains (Chevalier *et al.*, 2010). However, this class

[†]These authors contributed equally to this work.

[‡]Present address: Laboratoire des Matériaux Polymériques Multiphasiques (LMPMP), Faculté des Sciences de l'Ingénieur, Université Ferhat Abbas, Sétif, Algérie.

Abbreviations: EPI, efflux pump inhibitor; MDR, multidrug-resistant; PA β N, phenylalanine-arginine β -naphthylamide.

of compounds exposes a nitro group, representing a possible hazard, and we therefore planned to synthesize new molecules devoid of this disadvantageous functional group.

The aim of the present study was to evaluate the potential of new alkylaminoquinazoline derivatives to restore the drug susceptibility of *E. aerogenes*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* MDR strains. One compound exhibited a very efficient activity and restored significant susceptibility to various resistant clinical strains with respect to structurally unrelated antibiotics.

METHODS

Chemistry. The synthetic pathways of the alkylaminoquinazoline derivatives are shown in Fig. 1. Compounds were characterized by NMR spectrometry (^1H and ^{13}C NMR). Details of the synthesis and the purification process have been reported previously (Baitiche *et al.*, 2004).

Bacterial strains, growth conditions and antibiotic susceptibility tests. Three strains of *E. aerogenes*, CM-64, and clinical isolates EA5 and EA27, were used. CM-64 is a chloramphenicol-resistant strain that overexpresses the AcrAB pump, and isolates EA5 and EA27 exhibit energy-dependent norfloxacin and chloramphenicol efflux (Ghisalberti *et al.*, 2005; Mallaé *et al.*, 1998). Moreover, strain EA27 is an *acrR* mutant that overexpresses the AcrAB efflux pump (Lorenzi *et al.*, 2009). Two clinical strains of *K. pneumoniae*, KP55 and KPBj4, exhibiting an active efflux of norfloxacin and chloramphenicol, and one strain of *P. aeruginosa*, PA124, overexpressing OprM, the outer membrane component of the efflux system, were used (Lorenzi *et al.*, 2009; Chevalier *et al.*, 2000; Pagès *et al.*, 2009). *E. aerogenes* ATCC 13048, *K. pneumoniae* ATCC 11296 and *P. aeruginosa* PAO1 were used as references.

Bacteria were grown in Luria–Bertani (LB) or Mueller–Hinton (MH) broth at 37 °C. To determine MICs, approximately 10^6 cells were inoculated into 1 ml MH broth containing twofold serial dilutions of each antibiotic. The results were read after 18 h at 37 °C according to the Clinical and Laboratory Standards Institute (<http://www.clsi.org>) and the Comité de l'Antibiogramme de la Société Française de Microbiologie (<http://www.sfm.asso.fr/nouv/general.php?pa=2>). To

study synergistic activity, various concentrations of the potential inhibitors were added during the incubation with antibiotics. Control experiments were carried out without the inhibitors (Lorenzi *et al.*, 2009; Chevalier *et al.*, 2000).

Chloramphenicol uptake. Measurement of chloramphenicol uptake by intact cells has been described previously (Ghisalberti *et al.*, 2005; Mallaé *et al.*, 2002). Briefly, exponential-phase bacteria grown in LB broth were pelleted, washed once, and suspended to a density of 10^{10} c.f.u. ml^{-1} in 50 mM sodium phosphate/magnesium chloride. [^{14}C]chloramphenicol (Aventis Pharma) with a specific activity of 59.46 mCi mmol^{-1} (2.2 GBq mmol^{-1}) was added to the cell suspension at 37 °C in a shaking water bath, yielding a final concentration of 5 μM . At various intervals or after 2 min contact (for comparison studies), 100 μl of the suspension was removed and immediately filtered through a GF/C filter (Whatman). After three washes with 4 ml cold phosphate buffer containing 0.1 M LiCl, filters were dried and the radioactivity (c.p.m.) was measured in a Packard scintillation counter (PerkinElmer Life and Analytical Sciences). Quinazoline derivatives and PA β N were added 10 min before the radiolabelled antibacterial agent. Inhibition assays were performed in the presence of the quinazoline derivatives. Control samples without inhibitors were run under the same conditions (Chevalier *et al.*, 2008).

RESULTS

Effects of alkylaminoquinazoline compounds on various *E. aerogenes* strains

E. aerogenes ATCC 13048, the chloramphenicol-resistant variant CM-64, and EA5 and EA27, two clinical isolates which exhibit a MDR phenotype, were used to determine the intrinsic biological activity of various quinazolines (Fig. 1). The MICs of these compounds were particularly high (>15 mM towards the tested strains, for some products the threshold of solubility was about 15–20 mM) and similar to that obtained with PA β N (5 mM), an EPI currently used for the determination of efflux-mediated antibiotic resistance (data not shown). The determination of the MIC of each compound putatively defined as a potential EPI is an absolute requirement that assures the

Fig. 1. Synthetic pathway for the production of alkylaminoquinazoline compounds. The synthesis of the various quinazoline derivatives has been reported previously (Baitiche *et al.*, 2004). The structures of the compounds tested are shown together with their identification number (BG).

selection of concentrations that have no effect on the growth and viability of the strain.

The compounds were assayed, at concentrations that had no intrinsic effect on the growth of *E. aerogenes*, for their ability to decrease the level of chloramphenicol resistance of *E. aerogenes* strains EA27, EA5 and CM-64, all of which show active efflux of chloramphenicol. Among these compounds, 1167 was the most effective as an enhancer of chloramphenicol activity towards the tested resistant strains (Table 1). A divergence was noted with respect to the responses exhibited by the resistant strains, probably associated with variation in the respective strain physiology, e.g. modification of membranes (Chevalier *et al.*, 2008; Pagès *et al.*, 2009). In the case of strain EA5, no restoration of susceptibility was obtained with quinazoline compounds and only a weak effect was observed with PA β N. This may reflect the involvement of a membrane alteration previously reported in this strain (Malléa *et al.*, 1998) on the penetration and activity of the EPI or the presence of a chloramphenicol acetyltransferase.

In order to study further the EPI-like effect, 1167 was evaluated for its ability to increase the susceptibility of the ATCC 13048 and CM-64 strains to various quinolones. Whereas 1167 produced a great reduction of the MICs of nalidixic acid and ciprofloxacin in CM-64, the compound produced smaller but significant reductions of the MICs of norfloxacin and sparfloxacin (Table 2). However, 1167 had little if any effect on the MICs of norfloxacin, sparfloxacin or erythromycin in the ATCC reference strain. Because the presence of PA β N reduced the susceptibility of strain ATCC 13048 to nalidixic acid and erythromycin under the conditions used (Table 2), it may well be that this strain produces a PA β N-sensitive but quinazoline-insensitive efflux pump that is different from those overexpressed in the antibiotic-resistant strains affected by the quinazoline compounds. Moreover, this restoration conferred by PA β N upon macrolide susceptibility has been previously characterized in various strains of *E. aerogenes* and *Escherichia coli* (Chollet *et al.*, 2004). Regarding this point, it is interesting to note that no effect of 1167 on erythromycin

susceptibility was obtained, in contrast to PA β N (Table 2). In the resistant strain CM-64, 1167 was able to significantly decrease the MICs of ciprofloxacin and norfloxacin to the same extent as PA β N. Interestingly, the effect of 1167 on nalidixic acid and sparfloxacin activity was lower than that of PA β N.

Effect of quinazoline derivatives on chloramphenicol accumulation in *E. aerogenes* EA27 and CM-64

The effect of the alkylaminoquinazoline derivative 1167 on the intracellular accumulation of radiolabelled chloramphenicol was evaluated in the resistant EA27 and CM-64 strains, which overexpress the AcrAB-TolC efflux system (Ghisalberti *et al.*, 2005; Malléa *et al.*, 1998). Fig. 2 illustrates the kinetic accumulation of chloramphenicol in the CM-64 strain in the absence and presence of 1167. A strong increase was observed in the presence of 1167, indicating the effect of the compound on chloramphenicol efflux. An incubation time of 2 min was selected on the basis of the curve (Fig. 2) and previous data (Chevalier *et al.*, 2010). The effects of 1167 and 1187, a molecule containing a nitro group (Chevalier *et al.*, 2010), were assayed with strains CM-64 and EA27. At the same time, controls were carried out with PA β N, which is a well-characterized EPI (Chevalier *et al.*, 2010; Lomovskaya & Bostian, 2006). As is evident from Table 3, 1167 induced a noticeable increase of the intracellular concentration of labelled chloramphenicol in strain CM-64 and to a lesser extent in the clinical MDR isolate EA27, which overexpresses the efflux pump. Interestingly, the effects on chloramphenicol accumulation were quite similar with 1167 and 1187.

Effect of quinazoline derivatives on other Gram-negative resistant bacteria

Various resistant strains of *K. pneumoniae* and *P. aeruginosa* (Chevalier *et al.*, 2000; Pagès *et al.*, 2009) were tested with the compounds and with different antibiotic

Table 1. Effect of alkylaminoquinazolines on the chloramphenicol susceptibility of various *E. aerogenes* strains

See Fig. 1 for descriptions of the compounds. Values are the mean of three independent determinations.

Compound	Concentration (mM)	MIC of chloramphenicol (mg l ⁻¹)			
		ATCC 13048	CM-64	EA5	EA27
None		8	128	128	1024
1162	2.5	8	32	128	256
1163	2.5	8	32	128	256
1164	2.5	4	16	128	256
1165	2.5	4	16	128	256
1166	2.5	4	16	128	128
1167	2.5	8	8	128	128
PA β N	0.625	2	4	32	32

Table 2. Effects of alkylaminoquinazolines on the quinolone and erythromycin susceptibility of *E. aerogenes* ATCC 13048 and its resistant derivative CM-64

Abbreviations: Nal, nalidixic acid; Nfx, norfloxacin; Cpx, ciprofloxacin; Spx, sparfloxacin; Ery, erythromycin. Values are the mean of three independent determinations.

Compound (concentration, mM)	MIC (mg l ⁻¹)									
	ATCC 13048					CM-64				
	Nal	Nfx	Cpx	Spx	Ery	Nal	Nfx	Cfx	Spx	Ery
None	8	0.5	0.125	0.125	128	128	2	1	1	512
PAβN (0.100)	0.5	0.5	0.031	<0.007	<8	0.5	0.5	0.062	0.015	<8
PAβN (0.625)	0.5	0.25	0.015	<0.007	<8	0.5	0.25	0.062	<0.007	<8
1167 (2.500)	4	1	0.125	0.125	256	4	0.5	0.125	0.25	512

classes. With respect to the *K. pneumoniae* KP55 and KPBj4 isolates, a substantial increase in chloramphenicol, nalidixic acid and tigecycline susceptibilities was obtained with the compounds (Table 4). It is interesting to note that the level of restoration of antibiotic susceptibility obtained with 1167 and PAβN depends on the antibiotic class (related to their structural and functional properties) and on the presence of additional mechanisms of resistance such as target mutation, which can modulate the effect (Pagès & Amaral, 2009).

Evaluation of the compounds for their ability to reduce the resistance of *P. aeruginosa* strains to chloramphenicol and

nalidixic acid indicated that 1167 was effective (Table 4), although PAβN exhibited a greater restoration of activity. A similar effect was noted with ciprofloxacin (data not shown). These results suggest that 1167 is able to act on *P. aeruginosa* resistant strains and restore, though partially, the susceptibility to two different classes of antibiotics.

DISCUSSION

Multidrug resistance associated with bacterial efflux pumps presents a general healthcare problem, and in recent years, EPIs have been described as an attractive way to block antibiotic efflux and restore bacterial susceptibility. A major problem with this new family of antibacterial agents is the possible toxicity associated with the chemical structure of the compounds, which might impair development for future clinical use (Lomovskaya & Bostian, 2006; Pagès & Amaral, 2009). In this study, six alkylaminoquinazoline derivatives devoid of a nitro group were evaluated for their ability to reverse the antibiotic resistance of efflux-mediated MDR *E. aerogenes*. Among these derivatives, compound 1167 significantly increased the activity of chloramphenicol against a panel of resistant Gram-negative isolates. When 1167 was present during incubation with radiolabelled chloramphenicol of two chloramphenicol-resistant strains that overexpress the AcrAB efflux pump, an increase in the intracellular accumulation of the antibiotic was noted. These results demonstrate that this alkylaminoquinazoline restores the intracellular antibiotic concentration of *E. aerogenes* strains that overexpress efflux pumps.

In resistant strains of *K. pneumoniae*, *E. aerogenes* and *P. aeruginosa*, we observed a noticeable restoration of the activities of chloramphenicol and nalidixic acid, two antibiotics belonging to different classes which are substrates of the efflux pumps of these bacterial species (Li & Nikaido, 2009; Piddock, 2006; Poole, 2007). We have recently reported that other quinazoline derivatives also exhibit effects on resistant strains (Mahamoud *et al.*, 2007;

Fig. 2. Effect of compound 1167 on chloramphenicol accumulation in *E. aerogenes* CM-64. Exponential-phase bacteria grown in LB broth were removed, resuspended in sodium phosphate buffer and incubated with radiolabelled chloramphenicol for various times. Intracellular accumulation of chloramphenicol was followed in the absence (□) or presence of 2.5 mM 1167 (■). Values [expressed as 'UA', corresponding to c.p.m./OD₆₀₀ unit (Chevalier *et al.*, 2010)] were obtained from two independent experiments performed in duplicate. Error bars, SD.

Table 3. Effects of 1167 on the accumulation of labelled chloramphenicol

Values shown in parentheses indicate the SDs calculated from four independent experiments.

Compound (concentration)	Chloramphenicol accumulation (arbitrary units)	
	EA27	CM-64
None	9.5 (± 0.6)	1.3 (± 0.2)
PA β N (0.2 mM)	40 (± 7)	13 (± 2.1)
1167 (2.5 mM)	15 (± 0.9)	6.5 (± 0.4)
1187 (2.5 mM)*	19 (± 1)	7 (± 0.7)

*Compound previously described by Chevalier *et al.* (2010).

Chevalier *et al.*, 2010). The compound studied is able to restore the antibiotic susceptibility of *Enterobacteriaceae* and *P. aeruginosa*. This point is especially important considering that two major efflux pumps are expressed in these bacteria, i.e. AcrAB-TolC and MexAB-OprM (Davin-Régli *et al.*, 2008; Li & Nikaido, 2009; Chevalier *et al.*, 2008).

It has been previously determined that macrolides are expelled by *Escherichia coli* and *E. aerogenes* efflux pumps and that the efflux mechanism is inhibited by PA β N (Chollet *et al.*, 2004). Interestingly, 1167 is not able to restore erythromycin activity, unlike the other tested antibiotics, e.g. chloramphenicol and quinolones. This suggests that 1167 recognizes, at least partially, the same pump site as that used by quinolones and chloramphenicol and competes with them for transport. In contrast, the site or the selective pump involved in macrolide efflux (Yum *et al.*, 2009) is not recognized by 1167. The efficiency of restoration depends on the respective affinities of the transported drugs and the tested compound for affinity/binding sites located inside the efflux pump system. These sites have been proposed from the co-crystallographic analyses (Yu *et al.*, 2003, 2005) and modelling studies carried out on the AcrB pump (Eicher *et al.*, 2009; Li &

Nikaido, 2009; Murakami, 2008; Nikaido & Takatsuka, 2009; Pos, 2009; Takatsuka *et al.*, 2010). Consequently, as the concentration of the compound is increased, competition for these internal binding sites (Nagano & Nikaido, 2009) favours the compound, depending on the respective affinity constant ratio, thereby promoting the intrabacterial retention of the antibiotic. This effect results in the antibiotic reaching the levels necessary to ensure its activity towards the target.

Among the alkylaminoquinazoline derivatives, the tested compounds that contained a morpholine group associated with a propyl chain are more efficient than the others devoid of these groups. In addition, the nitro group located in the previous compound 1187 and representing a possible side-effect has been removed in compound 1167 without drastically altering the inhibitory ability. This point is especially important, since the development of an inhibitor of bacterial efflux pumps requires a low level of intrinsic activity combined with a reduced toxicity. Consequently, from these data, structure–activity relationship studies concerning this family of alkylaminoquinazoline derivatives may be fruitfully undertaken with the aim of identifying pharmacophoric groups involved in affinity site recognition.

Table 4. Effects of quinazolines on the antibiotic susceptibility of *K. pneumoniae* and *P. aeruginosa* strains

Abbreviations: Chl, chloramphenicol; Nal, nalidixic acid; Tig, tigecycline; KP, *K. pneumoniae* strain; PA, *P. aeruginosa* strain. Values are the means of three independent determinations.

Compound (concentration, mM)	MIC (mg l ⁻¹)												
	<i>K. pneumoniae</i> strain									<i>P. aeruginosa</i> strain			
	ATCC 11296			KP55			KPBj4			PAO1		PA124	
	Chl	Nal	Tig	Chl	Nal	Tig	Chl	Nal	Tig	Chl	Nal	Chl	Nal
None	4	16	0.25	32	>1024	2	128	128	2	256	>512	256	>512
PAβN (0.05)	1	1	0.25	2	128	0.5	4	0.5	0.25	1	0.5	1	0.5
1167 (0.625)	2	8	0.125	2	256	0.25	4	4	0.5	8	64	8	64

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