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Quinazoline derivatives are efficient chemosensitizers of antibiotic activity in *Enterobacter aerogenes*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* resistant strains

Jacqueline Chevalier ^{a,1}, Abdallah Mahamoud ^{a,1}, Milad Baitiche ^{a,2}, Elissavet Adam ^a, Miguel Viveiros ^{b,c}, Adriana Smarandache ^d, Andra Militaru ^{b,d}, Mihail L. Pascu ^{b,d}, Leonard Amaral ^{b,c}, Jean-Marie Pagès ^{a,b,*}

^a *UMR-MD1, Facultés de Médecine et de Pharmacie, Université de la Méditerranée, IFR88, Marseille, France*

^b *COST Action BM0701 (ATENS)*

^c *Unit of Mycobacteriology, UPMM, Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa, Lisbon, Portugal*

^d *National Institute for Laser, Plasma and Radiation Physics, Măgurele, Romania*

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* Corresponding author. Present address: UMR-MD1, Transporteurs Membranaires, Chimiorésistance et Drug Design, Faculté de Médecine, 27 Bd Jean Moulin, 13385 Marseille cedex 05, France. Tel.: +33 4 91 32 45 87; fax: +33 4 91 32 46 06.

E-mail address: jean-marie.pages@univmed.fr (J.-M. Pagès).

¹ These authors contributed equally to this work.

² Present address: Laboratoire des Matériaux Polymériques Multiphasiques (LMPMP), Faculté des Sciences de l'ingénieur, Université Ferhat Abbas, Sétif, Algérie.

ABSTRACT

Among the three series of quinazoline derivatives synthesised and studied in this work, some molecules increase the antibiotic susceptibility of Gram-negative bacteria presenting multidrug-resistant phenotypes. *N*-alkyl compounds induced an increase in the activity of chloramphenicol, nalidixic acid and sparfloxacin, which are substrates of the AcrAB-TolC and MexAB-OprM efflux pumps in clinical isolates. These molecules are able to increase the intracellular concentration of chloramphenicol in efflux pump-overproducing strains. Their activity depends on the antibiotic structure, suggesting that different sites may be involved for the recognition of substrates by a given efflux pump. Quinazoline molecules exhibiting a nitro functional group are more active, and structure–activity relationship studies may be undertaken to identify the pharmacophoric group involved in the AcrB and MexB affinity sites.

1. Introduction

Chemoresistance is an important health challenge concerning infectious diseases, and the worldwide dissemination of multidrug-resistant (MDR) bacteria has severely compromised the efficacy of our antimicrobial weapons and has dramatically increased the frequency of therapeutic failure [1]. The polyspecificity of bacterial efflux transporters that extrude antibiotics prior to reaching their intended target confers a general resistance phenotype that can enhance the acquisition of additional mechanisms of antibiotic resistance [2,3]. It has recently been demonstrated that expression of the AcrAB-TolC pump belonging to the resistance–nodulation–cell division (RND) transporter family plays an important role in the selection of target mutations in resistant Gram-negative bacteria [2–4]. Because of the clear involvement of RND transporters in the increased frequency of MDR clinical bacteria, efflux pumps are now considered as an attractive target for the development of antibiotic/efflux pump inhibitor (EPI) combinational therapy, similar to the current use of a β -lactam/ β -lactamase inhibitor mix [4]. Diverse compounds have been reported to alter the efflux activity of Gram-negative bacteria. Several strategies have been described for the selection of chemosensitizers that block the drug efflux capability of bacterial cells and reverse initial resistance to specific antibiotics [4].

Using a screening test that measures the response of antibiotic-resistant *Enterobacter aerogenes* strains, various compounds exhibiting some structural similarities to the quinolone family have been shown to restore the activity of antibiotics that are expelled by bacterial efflux pumps [4]. These quinoline derivatives are mainly active on MDR clinical isolates that overexpress efflux pumps. It has been

proposed that this family of molecules may compete with antibiotic transport within the AcrB pump [4].

The purpose of the present study was: (i) to assess the ability of new compounds to restore drug susceptibility of *E. aerogenes*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* MDR strains; and (ii) to determine the structure–activity relationships of these compounds. Three compounds were shown to exhibit a very efficient chemosensitizer profile and to restore significant susceptibility of various resistant clinical strains to structurally unrelated antibiotics.

2. Materials and methods

2.1. Chemistry

The synthetic pathways of the quinazoline derivatives have been reported previously [5].

2.2. Bacterial strains, growth conditions and antibiotic susceptibility testing

Enterobacter aerogenes CM-64, which is a chloramphenicol-resistant strain (ATCC 13048 derivative) overexpressing the AcrAB pump, and isolate EA27 exhibiting norfloxacin and chloramphenicol efflux were used [6,7]. Two *K. pneumoniae* isolates (KP4 and KP55) exhibiting active efflux of norfloxacin and chloramphenicol [6,7] as well as one strain of *P. aeruginosa* PA124 overexpressing OprM, the outer membrane channel of two efflux systems, were used [6,7]. *Enterobacter aerogenes* ATCC 13048, *K. pneumoniae* ATCC 11296 and *P. aeruginosa* PA01 were used as reference strains.

To determine minimal inhibitory concentrations (MICs), ca. 10^6 cells growing in Mueller–Hinton (MH) broth at 37 °C were inoculated into 1 mL of MH broth containing two-fold serial dilutions of each antibiotic. Results were read after 18 h at 37 °C according to the guidelines of the Clinical and Laboratory Standards Institute [8]. To study synergistic activity, various concentrations of the potential inhibitors were added during incubation with the antibiotics [6,7]. Control experiments were carried out without the different inhibitors.

2.3. Chloramphenicol uptake

Exponential-phase bacteria grown in Luria–Bertani broth were pelleted, washed once and suspended to a density of 10^{10} colony-forming units/mL in sodium phosphate buffer (50 mM, pH 7) containing 5 mM $MgCl_2$. [^{14}C]-chloramphenicol (Aventis Pharma, Romainville, France) was added to the cell suspension, yielding a final concentration of 5 μ M. The cell suspension was maintained at 37 °C for 2 min, then 100 μ L of the suspension was removed and immediately filtered through GF/C filters (Whatman International Ltd., Maidstone, UK). After three washes with 4 mL of cold phosphate/LiCl 0.1 M buffer, filters were dried and the radioactivity (counts per min) was measured in a Packard scintillation counter (Perkin-Elmer Life and Analytical Sciences, Wellesley, MA). Quinazoline derivatives and phenylalanine-arginine β -naphthylamide (PA β N) were added 10 min before [^{14}C]-chloramphenicol to perform inhibition assays.

2.4. Determination of compound stability

Stability studies were performed on compound 1188. The concentration of 1188 in solution ranged from 10^{-3} M to 10^{-6} M at pH 6.7. Solutions were kept in the dark and two cases were observed: (i) samples were kept at 4 °C and then measured at room temperature (22 °C) (when measured, they were first incubated for 30 min to reach room temperature); and (ii) samples were kept at room temperature. To rule out possible precipitation, the solutions were exposed to ultrasonic treatment (exposure time up to 15 min at 35 kHz with a peak output power of 240 W and HF power = 30 W_{eff}) [9]. Stability was checked by ultraviolet-visible absorption spectra measurements [9]. The stability of EPIs is a key point that has been reported by several studies focusing on molecules belonging to the peptidomimetic class [4]. Absorption measurements performed between 2×10^{-3} M and 10^{-6} M allowed computation of a polymerisation degree of 1.018, thereby indicating that at this concentration range there were insignificant changes in the stability of the compound during the tested period.

3. Results

3.1. Effect of quinazoline compounds on various *Enterobacter aerogenes* strains

Enterobacter aerogenes ATCC 13048, the chloramphenicol-resistant variant CM-64 and a clinical isolate EA27 [6,7] were used to determine the intrinsic biological activity of various quinazolines (Table 1). The MICs of these compounds were especially high and were similar to that obtained with PA β N, an EPI used for the determination of efflux-mediated antibiotic resistance in *P. aeruginosa* and *E. aerogenes* strains. Determination of the MIC for each compound is an absolute requirement that assures

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

Compounds were assayed at concentrations that have no intrinsic effect on their ability to decrease the level of chloramphenicol resistance of *E. aerogenes* EA27 and CM-64 expressing active efflux of chloramphenicol. Among these compounds, 1187, 1188, 1189 and 1190 were particularly effective as enhancers of chloramphenicol activity and reduced the MIC of chloramphenicol (Table 1). Although the individual responses of strains EA27 and CM-64 to each of the agents evaluated for an MIC, including the PA β N control, was identical, the degree of susceptibility to chloramphenicol is far greater for the CM-64 strain than for the EA27 strain. The activity of compounds for reducing susceptibility to chloramphenicol differed between the two strains and did not appear to be related to the degree of resistance to chloramphenicol. This is to be expected since the two strains have been shown to differ remarkably with respect to their membrane physiology characteristics [6,7].

To further study the EPI-like effect, molecules having the greatest effect on reduction of the MIC of chloramphenicol were evaluated for their ability to increase the susceptibility of ATCC 13048 and CM-64 to various quinolones. Whereas a great reduction of the MICs of nalidixic acid and ciprofloxacin was produced by 1188 and 1189 against CM-64, these compounds produced a lesser but significant reduction of the MIC of norfloxacin (Table 2). These compounds had little, if any, effect on the MIC of nalidixic acid, norfloxacin, sparfloxacin or erythromycin against the ATCC 13048 reference strain.

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

The effect of the quinazoline derivatives on intracellular accumulation of radiolabelled chloramphenicol by the chloramphenicol-resistant EA27 and CM-64 strains overexpressing the AcrAB-TolC efflux system [6,7] was evaluated. Controls with PA β N, the well known EPI [4], were conducted at the same time. Compounds 1187, 1188, 1189 and 1190 increased the level of intracellular chloramphenicol (Fig. 1). The effect of 1188 on intracellular accumulation of chloramphenicol by EA27 and CM-64 strains was dose-dependent, as was the case for the positive control PA β N (data not shown). It is interesting to check whether the quinazoline molecules are also able to increase the intracellular accumulation of another substrate of the AcrAB efflux pump. For this aim, the method recently developed to study ethidium bromide transport [10] was performed with *E. aerogenes* EA27 resistant strain. The result indicates that addition of 1189 significantly increased the intracellular concentration of ethidium bromide in this strain that overproduces the AcrAB pump (Fig. 1).

3.3. Effect of quinazoline derivatives on other Gram-negative resistant bacteria

Various resistant strains of *K. pneumoniae* and *P. aeruginosa* [6,7] were tested with the compounds in combination with different classes of antibiotics. An efficient increase of susceptibility to chloramphenicol and nalidixic acid was obtained with the compounds against *K. pneumoniae* KP4 isolate (Table 2). For KP55 isolate, the activity of chloramphenicol was improved, whereas a lesser effect was evident for nalidixic acid activity owing to the presence of target mutations. The compounds produced a similar reduction in the MIC of tigecycline against the two isolates (KP55 and KP4). This suggests that the compounds are also effective in reducing antibiotic

resistance of *K. pneumoniae* resistant isolates. It is interesting to note that the level of restoration of antibiotic susceptibility depends on the antibiotic class (related to their structural and functional properties) and the presence of additional mechanisms of resistance such as target mutation that can modulate the effect [4]. Quinazoline compounds had no effect on the antibiotic susceptibility of an *Escherichia coli* *acrAB*-deleted strain (data not shown), demonstrating that the variation in antibiotic susceptibility is related to overexpression of efflux pump activity.

The compounds were able to reduce resistance of *P. aeruginosa* strains to chloramphenicol and nalidixic acid (Table 2). A similar effect was also noted with ciprofloxacin (data not shown). These results suggest that the compounds, similarly to PA β N, are able to act on *P. aeruginosa* resistant strains and to restore, at least partially, susceptibility to two different antibiotic classes.

4. Discussion

In this study, 17 quinazoline derivatives were evaluated for their ability to reverse antibiotic resistance. Compounds 1187, 1188, 1189 and 1190 increased the activity of chloramphenicol against a panel of MDR Gram-negative isolates. The partial recovery of antibiotic susceptibility obtained with these compounds as well as with the EPI PA β N indicates that other non-efflux pump mechanisms, such as alteration of membrane permeability, target mutation etc., are responsible in part for the total antibiotic resistance of the strains [4]. An increase in intracellular accumulation of chloramphenicol is noted when the compounds are present during incubation of two chloramphenicol-resistant strains that overexpress the AcrAB efflux pump. In addition, the increase in intracellular concentration produced by compound 1188 in

the two resistant strains is dose-dependent. These results provide evidence that these quinazoline derivatives restore the intracellular antibiotic concentration in Gram-negative bacteria that overexpress efflux mechanisms.

The efficiency of restoration depends on the respective affinities of the transported drugs and the compounds for internal sites located inside the efflux pump; these sites have been proposed from crystallographic and modelling studies carried out with the AcrB pump [11,12]. A recent paper presented differences in the affinity constant for some antibiotics [13]. Consequently, as the concentration of a determined compound is increased, competition for these internal binding sites favours the compound depending on the respective affinity constant ratio, thereby promoting intrabacterial retention of the antibiotic. This effect results in increased antibiotic activity on the target.

We observed a significant increase in susceptibility to chloramphenicol and nalidixic acid, which are substrates of efflux pumps in *K. pneumoniae*, *E. aerogenes* and *P. aeruginosa* [2–4]. This is especially important taking into account the difference in the major efflux pumps expressed in these bacteria, e.g. AcrAB-ToIC and MexAB-OprM.

The difference in the restoration level obtained in the present study with the various quinazoline derivatives and the antibiotics tested may reflect: (i) a variation in the respective location of ligands inside the AcrB cavity such that binding of the compound interferes with the extrusion of the antibiotic; (ii) competition between the antibiotic and derivative for the same pump active site; (iii) the compounds differ with respect to the degree of affinity for a specific internal site; and (iv) binding of the

compound reduces the affinity of the antibiotic to a different site. The presence of different sites located inside the AcrB pump has been reported [14]. With the recent model illustrating functioning of the AcrB pump [11,12], it is possible that the same amino acid residues participated at different drug binding sites located in the pump tunnel. Moreover, several recent data on the *E. coli* AcrB efflux mechanism indicate that some amino acid residues are involved in the transport process to a varying extent during specific steps such as binding, transport, etc. [14–16]. This can explain the divergence observed in the competitive capacity of the compounds with drugs of different classes (chloramphenicol, quinolones, ethidium bromide) for binding sites within the pump type (AcrB, MexB) as well as the level of expressed efflux pump activity in the tested strains. Kinetic studies have reported variation in the affinity constant of various β -lactams for AcrB in *E. coli* cells [13]. This flexibility of the substrate site may be directly involved in the polyselectivity of the efflux pump; a site that can be modified by site-directed mutagenesis [15,16]. The role and overlap of affinity sites may explain the discrepancy observed in the increase of intracellular concentrations of different transported molecules such as chloramphenicol, quinolones and β -lactams. It is important to note that a chemosensitizer can restore a high level of intracellular antibiotic concentration, but it cannot bypass other resistance mechanisms such as target mutation or altered permeability. Moreover, some isolates may also overproduce other efflux pumps that are less susceptible to inhibitors [4].

Among the quinazolines derivatives, the tested compounds containing a nitro functional group that decreases the electronic density of the aromatic ring are more efficient than those that are devoid of this group. Structure–activity relationship

studies regarding homologous derivatives may be fruitfully undertaken to identify pharmacophoric groups involved in affinity site recognition. With the resolution of the three-dimensional structure of pump components and the recent models proposed for the pump function [11,12], development of efficient molecules that may restore the intracellular concentration of antibiotics in MDR bacterial pathogens is a promising way to circumvent efflux pump activity.

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Competing interests

None declared.

Ethical approval

Not required.

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Fig. 1. Intrabacterial accumulation of drugs and the effect of quinazoline compounds.

The intracellular concentration of radiolabelled chloramphenicol was followed in the absence or presence of phenylalanine-arginine β -naphthylamide (PA β N) (at 200 μ M) and the various quinazoline derivatives (used at 0.25 \times the respective minimal inhibitory concentration). Values (expressed as counts per min/optical density: arbitrary units) were obtained from independent duplicate measurements in *Enterobacter aerogenes* EA27 and CM-64 strains. Insert: Effect of compound 1189 on ethidium bromide intracellular concentration in strain EA27. Bacteria were grown in Mueller–Hinton broth overnight, washed twice in phosphate-buffered saline (pH 7.4) and their optical density adjusted to 0.6. Then, 0.95 μ L was transferred to microtubes containing 5 μ L of ethidium bromide (EB) at a concentration of 1.0 mg/L. The tubes were placed into a Corbett 3000 Thermocycler (Corbett Research, Sydney, Australia) programmed for 37 $^{\circ}$ C and 60 cycles each of 1 min. Fluorescence of EB in the medium is negligible and increases with time owing to the accumulation of EB within the periplasmic space. Details of the procedure have been published elsewhere [9]. White circles and black squares indicate EB accumulation in the absence and presence of 1189, respectively.

Table 1Activity of quinazoline compounds on *Enterobacter aerogenes* strains ^a

Molecule	Quinazoline MIC (mM)			Chloramphenicol MIC (mg/L)		
	ATCC 13048	CM-64	EA27	ATCC 13048	CM-64	EA27
0				8	128	1024
1181	5	5	5	4	16	256
1183	10	>5	>5	4	32	512
1184	5	5	5	4	64	256
1180	5	5	5	4	16	128
1185	5	>5	5	4	32	256
1182	10	5	5	8	32	256
1186	5	5	5	4	32	256
1174	10	>5	>5	4	32	256
1176	10	>5	>5	4	32	256
1177	10	>5	>5	4	64	512
1188	1.25	1.25	1.25	4	32	256
1189	2.5	5	5	2	32	256
1187	5	5	5	1	8	64
1193	>5 ^b	>5 ^b	>5 ^b	8 ^b	32 ^b	256 ^b
1190	2.5	2.5	2.5	2	32	256
1191	2.5	5	5	4	32	512
1192	10	10	10	2	64	512
PAβN	5	5	5	≤1	≤ 16	≤ 16

MIC, minimal inhibitory concentration; PAβN, phenylalanine arginine β-naphthylamide.

^a Values are the mean of three independent determinations.

^b Precipitates observed.

Table 2Effects of quinazolines on the antibiotic susceptibility of *Enterobacter aerogenes*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* strains ^a

Molecule	MIC (mg/L)																						
	<i>E. aerogenes</i>					<i>K. pneumoniae</i>							<i>P. aeruginosa</i>										
	ATCC 13048					CM-64					ATCC 11296		KP55		KP4			PA01		PA124			
	NAL	NFX	CPX	SPX	ERY	NAL	NFX	CPX	SPX	ERY	CHL	NAL	TIG	CHL	NAL	TIG	CHL	NAL	TIG	CHL	NAL	CHL	NAL
0	8	0.5	0.125	0.125	128	128	2	1	1	512	4	16	0.25	32	>1024	2	128	128	2	256	>512	256	>1024
PAβN	0.5	0.5	0.031	<0.007	<8	0.5	0.5	0.062	0.015	<8	1	1	0.25	2	128	0.5	4	0.5	0.25	<2	<2	<2	16
1187	4	0.25	0.062	0.125	512	16	0.25	0.125	0.25	512	2	8	0.5	4	512	0.25	16	8	0.5	16	64	128	1024
1188	4	0.25	0.062	0.062	256	8	0.25	0.062	0.125	512	2	16	0.25	4	1024	0.5	4	16	1	16	64	128	1024
1189	4	0.25	0.062	0.062	512	16	0.5	0.062	0.125	1024	1	4	0.25	4	256	0.25	16	8	0.5	16	32	64	1024
1190	4	0.25	0.031	0.062	512	16	0.25	0.125	0.25	512	4	8	0.5	2	1024	0.5	8	16	2	16	64	16	1024

MIC, minimal inhibitory concentration; NAL, nalidixic acid; NFX, norfloxacin; CPX, ciprofloxacin; SPX, sparfloxacin; ERY, erythromycin; CHL, chloramphenicol; TIG, tigecycline; PAβN, phenylalanine arginine β-naphthylamide.

^a Values are the mean of three independent determinations.

^b The various molecules are used at low concentrations against the respective bacterial strains (<0.25× the determined MICs).

