New Pyridoquinoline Derivatives as Potential Inhibitors of the Fluoroquinolone Efflux Pump in Resistant *Enterobacter aerogenes* Strains

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Received April 30, 2001

Enterobacter aerogenes, one of the most frequently isolated nosocomial pathogens in France, is exhibiting increasing multidrug resistance mechanisms associated with a change in membrane permeability. For drugs of the quinolone family, mutations in the target and active efflux play a prominent role in the resistance. We report here the effect of several pyridoquinoline derivatives that restore a noticeable fluoroquinolone accumulation to resistant strains that overexpress the MarA activator. Studies of the energy-dependent quinolone efflux indicate that the most efficient derivatives tested probably inhibit the resistance process by acting as substrate competitors on the pump extruding intracellular norfloxacin.

Introduction

Multidrug resistance (MDR) and similar systems have been reported in *Enterobacteriaceae*,[1−3] showing that this active protection system against toxic compounds is generally conserved.[4−8] Expression of the drug efflux mechanism is observed in resistant bacteria and is associated with a reduced drug uptake due to decrease in the permeability of the outer membrane.[4,6,9] The spread of several resistance mechanisms and modulation of the different bacterial responses to antibiotic therapy call for the study and characterization of MDR systems and the search for potential resistance-reversing agents that could restore antibiotic susceptibility in strains documented as resistant.

Enterobacter aerogenes is one of the most frequently described Gram-negative bacteria responsible for nosocomial respiratory tract infections.[10,11] We have previously shown that *E. aerogenes* clinical strains exhibiting altered outer membrane porins, irrespective of their association with an efflux process, are resistant to β-lactam and quinolone antibiotics.[12−14] The mechanisms of resistance found to drugs of the quinolone family are mutations of DNA gyrase or topoisomerase IV and the expression of an efflux pump that maintains intracellular concentrations at a level lower than the MIC.[15−17] Many studies have focused on the reversal of MDR in Gram-positive bacteria,[18−20] *Escherichia coli*,[21,22] and *Pseudomonas aeruginosa*.[23] The aim of the present work was initially to identify inhibitors of fluoroquinolone resistance in the context of MDR and subsequently to evaluate the ability of these compounds to inhibit norfloxacin efflux in resistant *E. aerogenes* strains. A resistant strain was constructed from a susceptible wild-type *E. aerogenes* strain, ATCC 13048, using the plasmid p9 that encodes the *E. coli marA* gene, which activates the MDR cascade.[3,4] The resulting strain, which exhibits the MDR phenotype, was used to analyze putative inhibitors of fluoroquinolone efflux pumps. The intracellular concentration of radiolabeled norfloxacin would be expected to rise if the pump was disabled by an inhibitor, resulting in a decrease in the minimun inhibitory concentrations of antibiotics in cases where efflux was the major mechanism of resistance.

Results

Screening for Reversers of Resistance in *E. aerogenes* Strains. Nine pyridoquinoline ethers and thioethers were assayed for their potential to increase fluoroquinolone susceptibility in the *E. aerogenes* strain ATCC 13048 containing the multicopy p9 plasmid expressing MarA[4] and other *E. aerogenes* strains exhibiting various phenotypes. The marA activator induces the expression of an efflux mechanism and also an increased synthesis of cytoprotective enzymes in conjunction with a decreased porin synthesis involved in MDR.[3] Consequently, when compared to the original ATCC 13048 strain, *E. aerogenes* ATCC 13048 p9 exhibited a 40-fold increase in resistance to norfloxacin (MIC of 2 vs 0.05 μg/mL for ATCC 13048) and a 20-fold increase in resistance to ciprofloxacin (MIC of 0.5 vs 0.025 μg/mL for ATCC 13048) (Table 1). In addition, the overproduction of MarA induced a serious decrease in porin expression, and a noticeable cephalosporin resistance was subsequently obtained in ATCC 13048 p9. The MIC for ceftazidime being 0.25 μg/mL in ATCC 13048 control cells was increased to 1 μg/mL for the p9 derivative strain, reflecting the impermeability effect on cephalosporin susceptibility. Regarding MarA activation of the efflux process, several compounds partially reversed the resistance to norfloxacin or ciprofloxacin by at least 2-fold at a concentration that had no antibacterial activity. The MICs of compounds tested alone were 64 μg/mL against *E. aerogenes* ATCC 13048 and its p9 derivative and 512−1024 μg/mL against *E. aerogenes* strain 3. As shown in Table 1, **2a** was significantly active at a concentration of 32 μg/mL when

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10.1021/jm010911z CCC: $20.00
Published on Web 10/11/2001

Table 1. MICs of Compounds Tested vs *E. aerogenes* Strains

		MICs (μg/mL)	
strain	regimen[a]	norfloxacin	ciprofloxacin
ATCC 13048	fluoroquinolone alone	0.05	0.025
	fluoroquinolone plus		
	1a (16μg/mL)	0.1	0.025
	1d (16μg/mL)	0.05	0.05
	1c (16μg/mL)	0.05	0.025
	1e (16μg/mL)	0.1	0.025
	lg (16μg/mL)	0.05	0.025
	2b (16μg/mL)	0.05	0.025
	2a (16μg/mL)	0.05	0.025
	2a (32μg/mL)	0.05	0.025
	2e (16μg/mL)	0.05	0.025
	2f (16μg/mL)	0.025	0.025
ATCC 13048, p9	fluoroquinolone alone	2	0.5
	fluoroquinolone plus		
	1a (16μg/mL)	1	0.25
	1d (16μg/mL)	2	0.5
	1c (16μg/mL)	1	0.05
	1e (16μg/mL)	1	0.25
	1g (16 μg/mL)	2	0.5
	2b (16μg/mL)	1	0.5
	2a (16μg/mL)	0.25	0.25
	2a (32μg/mL)	0.25−0.1	0.1
	2e (16μg/mL)	2	0.5
	2f (16μg/mL)	0.5	0.1
Ea 3	fluoroquinolone alone	128	32
	fluoroquinolone plus		
	1c (128μg/mL)	128	32
	1e (128μg/mL)	128	32
	2a (64μg/mL)	64	8
	2a (128μg/mL)	16	4

[a] Drugs, when tested alone, were active at 64 μg/mL concentration against *E. aerogenes* ATCC 13048 and its derivative p9 and were active at a concentration of 512 or 1024 μg/mL against *E. aerogenes* strain 3. Tests were performed using a checkerboard microtitration method. Values are means from two independent experiments.

tested in combination with norfloxacin and ciprofloxacin on ATCC 13048 p9 strain. In addition, the clinical *E. aerogenes* strain 3 that expresses an efflux system was studied.[13,14] In this case, **2a** was the only one found to be synergistic in restoring antibacterial effects of both norfloxacin and ciprofloxacin, an 8-fold decrease of respective MICs. The effect observed in the MarA-dependent *E. aerogenes* resistance is consistent with that of a blocker of the efflux resistance mechanism.

Norfloxacin Accumulation in *E. aerogenes* ATCC 13048 p9 and the Effect of 2a. To characterize the energy-dependent fluoroquinolone efflux, studies of norfloxacin accumulation in *E. aerogenes* ATCC 13048 p9 were performed using the method of Malléa et al.[13] In the absence of the uncoupler, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), the intracellular concentration of labeled norfloxacin was reduced to one-third to one-quarter of that observed in the original ATCC 13048 wild-type strain. Significant expulsion, which was sensitive to CCCP, was observed in strain ATCC 13048 p9 (Figure 2A). The intracellular accumulation increased rapidly over 30 s and reached a steady state after 5 min of incubation. The maximum level of norfloxacin, corresponding to about 140 ng/mg of protein, was obtained in the presence of CCCP. This quantity must be compared with the 35 ng/mg of protein observed in the absence of the uncoupler. These results indicate that the addition of CCCP, which collapses the efflux energy component, blocked the pump activity

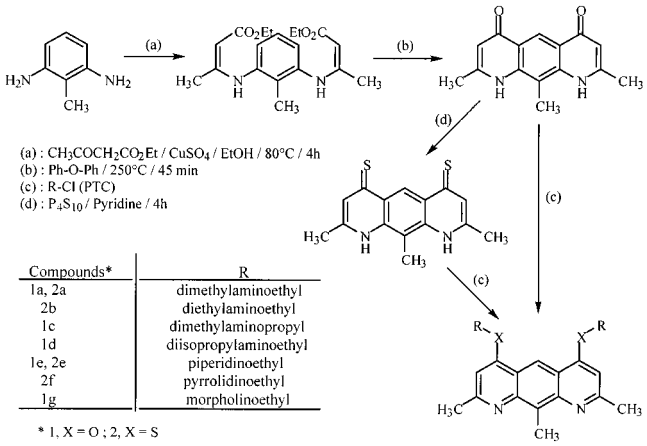

Figure 1. Synthetic pathways of the pyridoquinoline bis-ethers and bis-thioethers tested.

induced by MarA and allowed intracellular accumulation of norfloxacin. Thus, further study of various compounds as a putative efflux pump inhibitor is warranted.

2a decreased the quinolone MICs of *E. aerogenes* cells by altering drug efflux generated by MarA (see Table 1). To determine the exact effect, this compound was added to cell suspensions at final concentrations of 4, 0.8, 0.4, 0.2, and 0.04 mM prior incubation with labeled norfloxacin. Accumulation of norfloxacin was increased by a factor of 3.5 or 2.0 at 4, 0.8, and 0.4 mM, while no effects were noted at lower concentrations (Figure 2B). The effect of tested compound as blocker of the efflux pump (at 0.8 mM) was quite similar to that observed with the CCCP (at 50 μM) (Figure 2B). A dose-dependent response was observed with **2a** in conditions where the molecular ratio between compound and antibiotic was <100 (data not shown). The results suggest that the inhibition of the MarA-activated efflux pump is a competitive process.

Effects of Certain Compounds on the Norfloxacin Accumulation in *E. aerogenes* Isolate 3. The effect of three compounds (**1c**, **1e**, and **2a**) on the intracellular norfloxacin accumulation in *E. aerogenes* strain 3, a MDR clinical isolate,[13] was investigated (Figure 3). Preincubation of strain 3, which expresses an energy dependent efflux, with the compounds at a concentration of 0.8 mM increased the level of norfloxacin accumulation in the first few seconds. The final accumulation levels reached were 85%, 81%, and 69% of the intracellular norfloxacin obtained in CCCP-treated cells for **1c**, **1e**, and **2a**, respectively. These results therefore indicate that these compounds are probably acting as substrate competitors on the efflux pump and should have a similar effect on other clinical strain.

Discussion

It has recently been observed that a correlation exists between the antibiotic use in hospital wards and the emergence of strains with modified drug uptake characteristics,[24−26] i.e., membrane impermeability or efflux mechanisms.[6,12,26,27] This largely contributes to antibiotic resistance, which can result in adverse clinical outcomes and serious health problems.

E. aerogenes, the third leading cause of Gram-negative respiratory tract nosocomial infections, exhibits

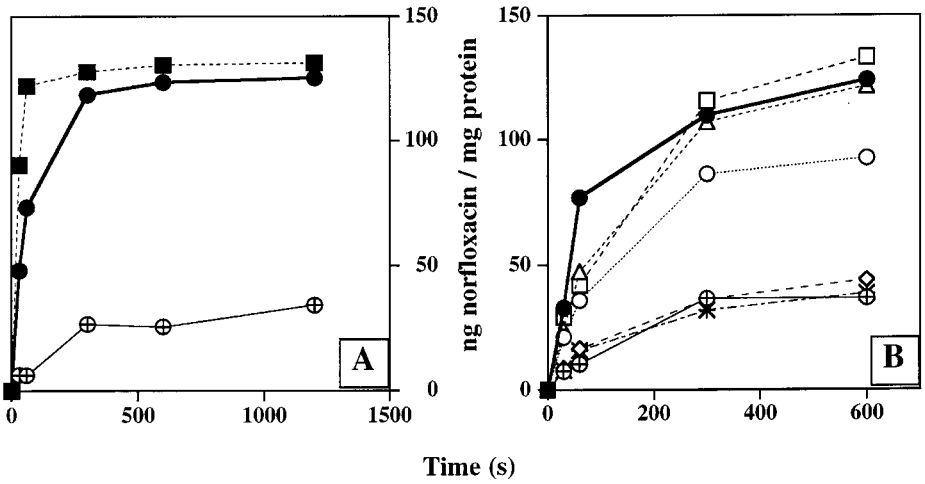

Figure 2. Norfloxacin accumulation in *E. aerogenes* ATCC 13048 and its p9 derivative. (A) Accumulation of radiolabeled norfloxacin with the strain ATCC 13048 in the absence of CCCP (■) and with its derivative p9 in the absence (⊕) and in the presence of CCCP (●). Values are means from two independent experiments. (B) Effect of **2a** on the norfloxacin accumulation in *E. aerogenes* ATCC 13048, p9. The uptake of radiolabeled norfloxacin was evaluated after 10-min preincubation with no compound (⊕) and with **2a**: 0.04 (*), 0.2 (◇), 0.4 (○), 0.8 (Δ), and 4 mM (□) or with 50 μM CCCP (●). Values are means from two independent experiments.

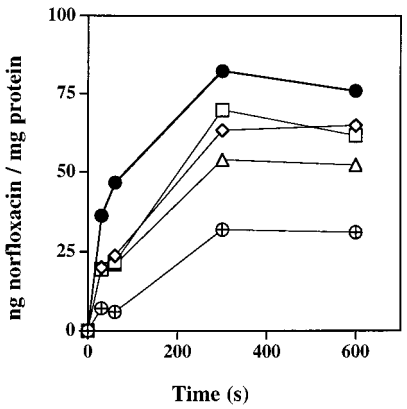

Figure 3. Effect of three pyridoquinoline derivatives on the norfloxacin accumulation in *E. aerogenes* isolate 3. The uptake of radiolabeled norfloxacin was evaluated after 10-min preincubation with no compound (⊕) or with **1c** (◇), **1e** (□), and **2a** (Δ) at 0.8 mM concentration or with 50 μM CCCP (●).

high resistance to broad spectrum antibiotics. We recently described the presence of efflux-mediated quinolone resistance in various clinical isolates.[13] In several strains, this mechanism is associated with decreased synthesis of outer membrane porins and increased β-lactamase-mediated resistance. The *mar* regulon characterized in *E. coli* and *Salmonella typhimurium* and recently described in *Shigella flexneri, Klebsiella pneumoniae,* and *E. aerogenes*[28,29] plays a central role in governing the expression of such bacterial responses. By using the MarA activator, which increases the expression of the AcrAB-TolC pump and decreases porin synthesis,[4] the identification of inhibitors of the efflux pump mechanism is of particular interest regarding the restoring intracellular antibiotic concentration. Several pyridoquinolines were assayed in the presence of norfloxacin and ciprofloxacin using resistant *E. aerogenes* strains. Among these derivatives, 4,6-bis(dimethylaminoethylthio)pyrido[3,2-*g*]quinoline (**2a**) and 4,6-bis(pyrrolidinoethylthio)pyrido[3,2-*g*]quinoline (**2f**) (to a lesser extent) were effective in restoring fluoroquinolone accumulation and partial susceptibility in the strain expressing MarA-mediated efflux process. The *mar* cascade[3] generates a decrease of porin expression, which is a major way for fluoroquinolone uptake.[30] Consequently, inhibitor of efflux mechanism cannot restore additional steps included in the resistance strategy, porin defficiency, target mutation, ..., and we observe only a partial recovery of susceptibility. Concerning the clinical isolate **3**, the reversers simultaneously induce a partial recovery of quinolone susceptibility, an 8-fold decrease of MICs with **2a**, and a significant increase in norfloxacin accumulation. This result suggests that the porin alteration and the mutation in the QRDR domain of gyrase, previously emphasized by Malléa et al.,[13] modulate the potentiating efficiency of the synergy.

2a produces a noticeable effect on the norfloxacin intracellular concentration, both in the *marA*-transformed strain and in the clinical isolate. This response profile may be associated with a competitive effect that occurs during the active pumping out of the norfloxacin. The efficiency of the restoration depends on the respective affinities of the transported drug and the competitor for the pump system involved in the efflux. Further studies will be necessary in order to determine precise activity as a competitor of these compounds and to assay them with members of other antibiotic families and other Gram-negative bacteria exhibiting a variety of distinct resistance phenotypes.

Experimental Section

Chemistry. 4,6-Bis(alkoxy)- and 4,6-bis(thioalkoxy)pyrido-[3,2-*g*]quinolines were prepared using the synthetic pathways (Figure 1) and previously reported methods.[31,32] Crudes were purified by crystallization. Purity was checked by thin-layer chromatography. Compounds were characterized by nuclear magnetic resonance spectrometry (^{1}H and ^{13}C NMR).

Bacterial Strains and Growth Conditions. *E. aerogenes* ATCC 13048 and its derivative strain containing the p9 plasmid that harbors the *E. coli* functional gene *marA*[4] were used. *E. aerogenes* **3** is a clinical isolate previously reported to exhibit energy-dependent norfloxacin efflux.[13,14] Bacteria were routinely grown in Luria-Bertani or Muller-Hinton broth at 37 °C. For the strain containing p9, the medium was supplemented with tetracycline (15 μg/mL).

Screening by Synergy Testing and Measurement of the Norfloxacin Uptake. Synergy testing was performed by checkerboard titration in microtiter plates. Nine new pyridoquinoline ethers and thioethers were assessed at 0.25, 1, 4, and 16 μg/mL for reversal of resistance to norfloxacin and ciprofloxacin in two *E. aerogenes* strains, ATCC 13048 and its p9 derivative. Norfloxacin and ciprofloxacin were added to final concentrations ranging from 0.005 to 0.1 μg/mL and from 0.0025 to 0.05 μg/mL, respectively, for *E. aerogenes* ATCC 13048 and from 0.1 to 2 μg/mL and 0.025 to 0.5 μg/mL, respectively, for the p9 derivative. Assays were performed on the resistant clinical strain *E. aerogenes* **3** with three compounds at concentrations ranging from 8 to 128 μg/mL in combination with norfloxacin and ciprofloxacin added to a final concentration ranging from 16 to 128 μg/mL and from 4 to 64 μg/mL, respectively. Wells were inoculated to the final concentration of 10^5 cells/mL. Microplates were assessed visually for growth after an 18-h incubation period at 37 °C.

The uptake of [^{14}C]norfloxacin by intact cells was previously described by Malléa et al.[13] To de-energize the bacteria, 50 μM CCCP was added 10 min before the radiolabeled antibacterial agent. Inhibition assays were performed in the presence of different amounts of the chemical compounds to obtain the final inhibitor concentrations of 4, 0.8, 0.4, 0.2, and 0.04 mM, after addition to the cell suspension. Control samples were run in the same conditions. The protein concentration was routinely determined with the BCA protein assay (Pierce, Rockford, IL).

Acknowledgment. We thank C. Bollet, A. Crémieux, and A. Davin-Regli for discussions, and we gratefully acknowledge M. Malléa for helpful advice. Plasmid p9 was a gift from P. Miller (Parker-Davis Pharmaceutical Research, Ann Arbor, MI). Labeled norfloxacin was a generous gift from Merck Sharp & Dohme Laboratories. This study was supported by the Université de la Méditerranée, the Institut National de la Santé et de la Recherche Médicale, the Centre National de la Recherche Scientifique, and the PACA région. This work was partly carried out in accordance with the "Cost B16 Action".

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JM010911Z