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Effect of 1-(1-naphthylmethyl)-piperazine, a novel putative efflux pump inhibitor, on antimicrobial drug susceptibility in clinical isolates of Enterobacteriaceae other than *Escherichia coli*

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***Objectives*: 1-(1-Naphthylmethyl)-piperazine (NMP) has been shown to reverse multidrug resistance (MDR) in *Escherichia coli* overexpressing RND type efflux pumps, but there is no data on its activity in Enterobacteriaceae other than *E. coli*.**

***Methods*: The antimicrobial susceptibilities of laboratory strains and 167 clinical isolates of Enterobacteriaceae to a variety of antimicrobial agents were determined in the absence and presence of NMP and, for comparison, of Phe-Arg-β-naphthylamide (PAβN), another putative efflux pump inhibitor (EPI). A 4-fold or greater reduction of the MIC after EPI addition was considered significant.**

***Results*: NMP consistently reduced the MIC of linezolid in *Citrobacter freundii*, *Enterobacter aerogenes* and *Klebsiella pneumoniae* clinical isolates. Significant effects of NMP addition in >50% of tested isolates were also seen for levofloxacin, tetracycline and chloramphenicol in *E. aerogenes*, and for levofloxacin and tetracycline in *K. pneumoniae*, whereas no or minor effects were observed in *Serratia marcescens*. MDR reversal by NMP was more likely in isolates with decreased susceptibility to fluoroquinolones. In most fluoroquinolone-resistant strains the activity was sufficient to render isolates drug-susceptible at clinically achievable concentrations. The activity of PAβN was different from that of NMP, suggesting different modes of action of the two putative EPIs.**

***Conclusion*: NMP has moderate activity in reversing MDR in many but not all members of the Enterobacteriaceae family including clinical isolates. Its effects on resistance reversal depend on bacterial species and drug, and are different from those seen with PAβN.**

Keywords: multidrug resistance, fluoroquinolones, nosocomial pathogens

Introduction

Multidrug resistance (MDR) has been an emerging problem among many bacterial pathogens, notably among nosocomial Gram-negative bacteria. An important mechanism of MDR in Gram-negative bacteria is enhanced efflux of chemically unrelated agents, thereby diminishing access of drugs to their intracellular targets.[1] MDR phenotypes can be selected both *in vitro* and *in vivo* in a variety of bacterial species by exposure to fluoroquinolones, a relatively new class of highly potent antibacterial compounds that are now being widely used in human and veterinary medicine. This type of MDR has often been linked with overexpression of resistance-nodulation-cell division (RND) type tripartite efflux pumps.[1]

A new development is the application of putative efflux pump inhibitors (EPIs) that are capable of at least partially reversing the MDR phenotype of Gram-negative bacteria by mechanisms not clearly understood.[2] One of these compounds with EPI activity in *Pseudomonas aeruginosa* is Phe-Arg-β-naphthylamide (PAβN).[3,4] Several arylpiperidines and other compounds capable

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of reversing MDR in defined Enterobacteriaceae have also been described.[5–8] Recently, 1-(1-naphthylmethyl)-piperazine (NMP) has been shown to reverse MDR in *Escherichia coli* overexpressing RND type efflux pumps, but not in pump-deficient mutants.[9] NMP increased the intracellular concentration of ethidium bromide and levofloxacin, suggesting efflux pump inhibition as the mechanism of action. In the present work we evaluated the potency of NMP to reverse MDR in several species of Enterobacteriaceae other than *E. coli*, including clinical isolates.

Materials and methods

Bacterial strains

Clinical non-duplicate isolates of *Citrobacter* spp., *Klebsiella pneumoniae*, *Enterobacter* spp. and *Serratia marcescens* were obtained from different clinical microbiology laboratories. Most isolates were from patients admitted to intensive care units. No attempts were made to investigate in detail the nature of MDR phenotypes among the clinical test isolates. Thirty out of the 167 isolates (18%) were fluoroquinolone-resistant (FQR, MIC of levofloxacin ≥8 mg/L), and 52 had an MIC of levofloxacin >1 mg/L. Reference strains of Enterobacteriaceae were from the ATCC. Laboratory strains included *Citrobacter freundii* CFAS0 that was obtained from *C. freundii* ATCC 8090 by inactivating *acrB* using the phage lambda-based Red/ET homologous recombination system (Gene Bridges, Dresden, Germany).[10] *Enterobacter aerogenes* EAEP298 was derived from a clinical isolate by *tolC* inactivation and was kindly donated by Jean-Marie Pagès.[11] *E. coli* 261, a gift from Jing Chen, carried *sdeXY* which codes for an RND type MDR efflux pump of *S. marcescens*.[12]

Chemicals and media

NMP was obtained from Chess GmbH (Mannheim, Germany), and PAβN and pyronin Y were purchased from Sigma-Aldrich (Steinheim, Germany). Luria–Bertani (LB) broth and agar were obtained from Oxoid (Basingstoke, UK). Ethidium bromide was from Merck (Darmstadt, Germany).

Susceptibility testing

Susceptibilities to a panel of different antibiotics were studied by microbroth dilution in the presence or absence of NMP or PAβN, in accordance with NCCLS performance and interpretive guidelines. Custom microtitre plates containing selected antimicrobials at increasing concentrations were purchased from Merlin Diagnostics (Bornheim, Germany). A 4-fold or greater reduction in the MIC values after addition of NMP or PAβN was considered significant.

Pyronin Y whole cell accumulation assays

Cells were grown overnight on LB agar plates and diluted in 1 mL of PBS + 0.4% glucose (pH 7.4) until an optical density at 600 nm of ∼1 was reached. The cells were then transferred to a 96-well plate and NMP was added. Thereafter, pyronin Y was added to a final concentration of 5 or 10 mg/L, and the relative fluorescence intensity was measured over time in a Safire (Tecan, Crailsheim, Germany) fluorescence plate-reader (excitation 545 nm, emission 570 nm). This assay was similar to a previously described ethidium bromide accumulation test,[9,13] but, unlike with ethidium bromide, the fluorescence of pyronin Y is quenched after binding to RNA, and decreasing fluorescence correlates with increasing intracellular dye concentration (and efflux inhibition). The use of pyronin Y instead of ethidium bromide as fluorescent dye offered the advantage to include PAβN as EPI, which showed no or only minor effects on the reduction of ethidium bromide MIC in reference strains of Enterobacteriaceae other than *E. coli* (Table 1), *E. coli* clinical isolates (W. V. Kern and P. Steinke, unpublished observations) and *P. aeruginosa*.[3]

Results and discussion

Intrinsic antibacterial activity of NMP and PAβN

At the concentration needed to reduce the MIC of levofloxacin and other agents by at least 4-fold in *E. coli* overexpressing *acrAB* or *acrEF* (between 50 and 100 mg/L),[9] NMP had no measurable growth inhibitory effects (data not shown) even in the pump-deficient strains *C. freundii* CFAS0, *E. aerogenes* EAEP298 and *E. coli* 262. This was unlike PAβN that had increased activity in pump-deficient strains (MIC ≤ 100 mg/L) compared with wild-type parental strains and clinical isolates (MIC > 400 mg/L). In clinical isolates, the intrinsic antibacterial activity of NMP was similar to that seen in reference strains (MIC ≥ 400 mg/L).

EPI activity in reference and laboratory strains

When used at a concentration of 25 mg/L, NMP had minor effects on the MIC of the test drugs in reference strains (data not shown)—similar to that seen earlier in *E. coli*.[9] At a concentration of 100 mg/L consistent effects were observed on ethidium bromide resistance reversal (Table 1). Effects on the MIC of the other agents were variable. Effects were generally less significant in *S. marcescens* and *E. coli* 261 (which carried the *S. marcescens* specific *sdeXY* pump gene) than in the other species (Table 1). Notably, in pump-deficient strains, NMP was unable to reduce the MICs of the test agents except that of rifampicin, whereas PAβN obviously was more toxic presumably owing to its enhanced intrinsic activity in RND type pump-deficient versus pump-competent strains. There was an exceptional interaction across all strains between PAβN and rifampicin in the sense of a highly sensitizing effect of PAβN that was largely independent of RND pump and *tolC* inactivation (Table 1).

There were small but reproducible effects of PAβN at a concentration of 25 mg/L on pyronin Y MICs in the tested Enterobacteriaceae strains that were not seen with ethidium bromide (Table 1). This provided the opportunity to assess the effects of both putative EPIs on intracellular substrate accumulation. Consistent with the observations of MIC changes there were measurable effects of both putative EPIs on pyronin Y fluorescence in *Citrobacter*, suggesting that the MIC changes after EPI addition were in fact related to changes in the intracellular substrate concentration (Figure 1). In *E. aerogenes* EAEP289, changes in the intracellular pyronin Y accumulation after NMP addition could also be demonstrated, but these changes were smaller, and a definite effect on increased pyronin Y accumulation after addition of PAβN was uncertain (Figure 1).

EPI activity in clinical isolates

As shown in Table 2, the spectrum of antimicrobial drugs affected by the addition of NMP (100 mg/L) depended on species and drugs. Across the different species, no effects of NMP were seen with streptomycin (as expected), and limited effects were seen with oxacillin, a relatively hydrophobic β-lactam (data not shown), and clarithromycin. Conversely, NMP consistently reduced the

Table 1. MICs of dyes and selected antibacterial agents in the presence and absence of NMP (100 mg/L) and PAβN (25 mg/L) in reference and laboratory strains

	MIC (mg/L)									
Strain	ethidium bromide	pyronin Y	levofloxacin	tetracycline	chloramphenicol	rifampicin	clarithromycin	linezolid	oxacillin	streptomycin
C. freundii ATCC 8090	256	128	0.063	0.5	2	16	128	256	128	4
+NMP	**64**	**32**	0.031	0.5	1	**4**	128	**16**	**32**	2
+PAβN	256	64	**0.016**	0.5	1	**0.063**	**1**	**16**	**16**	2
C. freundii CFAS0 (ΔacrB)	8	2	0.016	0.5	1	16	4	8	1	1
+NMP	8	2	0.016	0.25	1	**2**	2	8	0.5	1
+PAβN	8	2	0.016	0.25	0.5	**0.063**	**0.5**	**0.5**	0.5	1
E. aerogenes ATCC 13048	1024	256	0.125	4	4	32	256	256	128	4
+NMP	**256**	**64**	**0.031**	**1**	**1**	**8**	128	**32**	**32**	8
+PAβN	1024	128	0.063	4	**1**	**0.25**	**2**	**32**	64	4
E. aerogenes EAEP289	1024	256	32	8	256	16	512	1024	512	128
+NMP	**256**	**32**	**4**	**1**	**64**	8	256	**16**	**128**	256
+PAβN	1024	128	**8**	4	**64**	**0.5**	**8**	**64**	**128**	256
E. aerogenes EAEP298 (ΔtolC)	32	16	1	0.5	32	8	4	8	256	128
+NMP	16	8	2	0.25	32	**2**	2	8	128	256
+PAβN	16	8	2	0.5	32	**0.125**	**0.5**	4	128	256
K. pneumoniae ATCC 13833	512	64	0.125	2	4	16	128	256	64	4
+NMP	**64**	32	**0.031**	**0.5**	**1**	8	128	**8**	32	4
+PAβN	256	32	0.063	2	**1**	**0.125**	**2**	**32**	32	2
S. marcescens ATCC 14756	1024	128	0.25	16	4	32	256	128	256	256
+NMP	**256**	64	0.25	8	4	16	256	128	256	256
+PAβN	1024	128	0.25	16	4	**2**	128	64	128	256
E. coli 261 (262 carrying *sdeXY*)	128	8	0.25	1	128	16	64	128	32	16
+NMP	**16**	4	0.25	1	64	16	64	128	64	64
+PAβN	64	4	0.25	1	128	**1**	**16**	64	16	32
E. coli 262 (ΔacrB)	8	0.5	0.063	0.25	0.5	8	1	8	2	4
+NMP	4	4	0.031	0.25	1	**2**	1	16	1	4
+PAβN	4	0.5	**0.016**	0.25	0.5	**0.063**	0.5	4	**0.5**	2

A reduction of at least 4-fold is indicated in boldface.

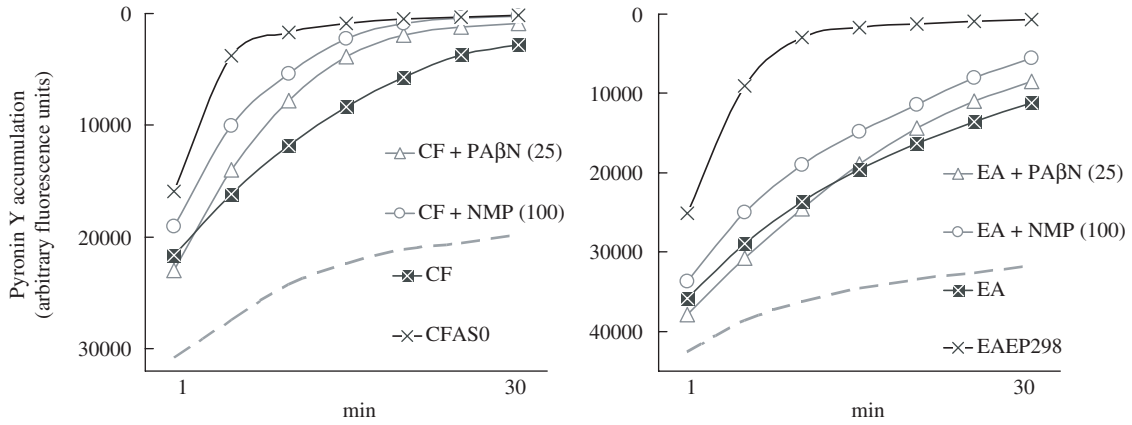

Figure 1. Intracellular concentration of pyronin Y as measured in a whole cell accumulation assay over time in the absence and presence of NMP (100 mg/L) and PAβN (25 mg/L) in *C. freundii* ATCC 8090 (= CF) and its *acrB*$^-$ mutant (CFAS0), and in *E. aerogenes* EAEP289 (= EA) and its *tolC*$^-$ mutant (EAEP298). The dashed line represents the background (pyronin Y fluorescence). Decreasing fluorescence corresponds to increasing intracellular concentration. Pyronin Y was added at a concentration of 5 mg/L (*Citrobacter*) or 10 mg/L (*Enterobacter*) at time 0.

Table 2. Effects of the putative EPIs NMP and PAβN on the MICs of different antimicrobial agents in clinical isolates of Enterobacteriaceae other than *E. coli*

			No. of isolates (%) with indicated fold reduction of MIC after addition of			
			NMP (100 mg/L)		PAβN (25 mg/L)	
Species (*n*)	Drug	MIC_{50} (mg/L)	≥4-fold	≥16-fold	≥4-fold	≥16-fold
C. freundii (34)	levofloxacin	0.125	13 (38%)	1	14 (42%)	–
	tetracycline	1	8 (24%)	–	12 (36%)	–
	chloramphenicol	4	10 (29%)	–	14 (42%)	–
	linezolid	256	33 (**97%**)	29 (**85%**)	30 (**88%**)	18 (**55%**)
	clarithromycin	128	–	–	32 (**94%**)	30 (**91%**)
	rifampicin	16	21 (**62%**)	–	34 (**100%**)	33 (**97%**)
E. aerogenes (25)	levofloxacin	0.25	21 (**84%**)	2	11 (44%)	2
	tetracycline	4	18 (**72%**)	1	7 (28%)	–
	chloramphenicol	4	17 (**68%**)	3 (12%)	15 (**60%**)	1
	linezolid	256	24 (**96%**)	23 (**92%**)	25 (**100%**)	18 (**72%**)
	clarithromycin	256	4 (16%)	1	25 (**100%**)	24 (**96%**)
	rifampicin	32	13 (**52%**)	–	25 (**100%**)	25 (**100%**)
K. pneumoniae (38)	levofloxacin	1	29 (**76%**)	1	15 (39%)	1
	tetracycline	4	28 (**74%**)	1	7 (18%)	–
	chloramphenicol	16	16 (42%)	5 (13%)	13 (34%)	2
	linezolid	512	38 (**100%**)	32 (**84%**)	36 (**95%**)	17 (43%)
	clarithromycin	128	–	–	38 (**100%**)	35 (**92%**)
	rifampicin	32	23 (**61%**)	2	38 (**100%**)	38 (**100%**)
S. marcescens (70)	levofloxacin	0.25	2 (3%)	–	2 (3%)	–
	tetracycline	8	34 (49%)	1	–	–
	chloramphenicol	4	11 (16%)	2	6	1
	linezolid	128	25 (36%)	1	25 (36%)	1
	clarithromycin	256	–	–	3	2
	rifampicin	32	2	–	64 (**91%**)	49 (**70%**)

Percentages are in boldface if >50%.

MIC of linezolid in the majority of *C. freundii*, *E. aerogenes* and *K. pneumoniae* clinical isolates, but this interaction did not result in a clinically relevant reduction of the MIC to levels below the breakpoint of resistance. Significant effects of NMP addition in >50% of tested clinical isolates were also seen for levofloxacin, tetracycline and chloramphenicol in *E. aerogenes*, and for levofloxacin and tetracycline in *K. pneumoniae*. No or minor effects were observed in *S. marcescens* (Table 2) including ethidium bromide.

In many FQR strains (five out of seven *Citrobacter* isolates, six out of six *Enterobacter* isolates and three out of three *Serratia* isolates) the activity of NMP was sufficient to render isolates drug-susceptible at clinically achievable concentrations. However, this was dependent on the initial MIC level. In FQR *K. pneumoniae* isolates, for example, which were almost all highly FQR (levofloxacin MIC >32 mg/L), addition of NMP was insufficient to decrease the MIC of levofloxacin to <8 mg/L (data not shown). Although clarithromycin was a pump substrate given the MIC changes in pump-deficient strains, the effects of NMP on its MIC were small and usually non-significant (Table 2).

PAβN showed a slightly differing spectrum of activity (Table 2). Particularly strong effects across the species were observed for the interaction of PAβN and rifampicin, similar to those seen in *E. coli*.[9] Apart from these effects, PAβN significantly and more effectively than NMP reduced the resistance to clarithromycin in all species except *S. marcescens* (Table 2), which showed very minor effects of PAβN on increased drug susceptibility. Effects of PAβN on macrolide resistance were previously described in *E. coli* and *E. aerogenes*, and were linked to the AcrAB-TolC system, but PAβN may be additionally active on a pump with high specificity for macrolide and ketolides[14] while NMP was found unable to reduce the MIC of ketolides in *E. coli*.[9] It is tempting to speculate that the strong effects of PAβN on macrolide resistance observed here in Enterobacteriaceae other than *E. coli* are linked to dual inhibition of an RND type efflux pump and a pump that preferentially accommodates macrolides/ketolides. A permeabilizing effect of PAβN, however, which may specifically affect rifampicin and macrolides (as shown for polymyxin B nonapaptide) cannot be excluded.

PAβN at a concentration of 25 mg/L had very limited effects on fluoroquinolone MICs (Table 2), but at a concentration of 100 mg/L it enhanced the effect on the reduction of the levofloxacin MIC to a potency that was similar to NMP (data not shown).

The subgroup of 52 clinical isolates with decreased fluoroquinolone susceptibility (and usually MDR phenotypes) were characterized in general by more significant EPI effects. In this subgroup PAβN even at the higher concentration of 100 mg/L had no effect on the MIC of ethidium bromide. It significantly reduced the pyronin Y MIC in most isolates of *C. freundii*, but much less so in *E. aerogenes*, *S. marcescens* and *K. pneumoniae*. NMP, in contrast, was more effective than PAβN in reducing the MICs of both dyes (data not shown).

Conclusions

Taken together, NMP showed moderate activity as an MDR reversal agent in Enterobacteriaceae other than *E. coli*. Resistance reversal by NMP to clinically relevant MIC values was achieved for levofloxacin in a variable proportion of the clinical isolates. The spectrum of activity of NMP differed from that seen with PAβN. PAβN was consistently associated with a large reduction of rifampicin MICs across the species tested, and with large reductions of clarithromycin MICs in all species except *S. marcescens*. Another noteworthy difference was the differential ability of the two EPIs to reduce the MICs of the two dyes, ethidium bromide and pyronin Y. This suggests the presence of different targets of the two putative EPIs within a given species. Alternative explanations include different binding sites in a given pump, and perhaps other effects such as differential membrane permeability alterations by one of the two compounds.

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Transparency declarations

None to declare.

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