

Selected Arylpiperazines Are Capable of Reversing Multidrug Resistance in *Escherichia coli* Overexpressing RND Efflux Pumps

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Several arylpiperazines capable of reversing multidrug resistance (MDR) in *Escherichia coli* overexpressing *acrAB* and *acrEF* but not in pump-deficient mutant strains were identified. 1-(1-Naphthylmethyl)-piperazine, one of the more active compounds, enhanced susceptibility to fluoroquinolones and other agents and increased the intracellular concentration of levofloxacin and ethidium bromide, suggesting efflux pump inhibition as the mechanism of MDR reversal.

Bacterial resistance to chemically unrelated antimicrobial agents (multidrug resistance [MDR]) may be caused by overexpression of MDR efflux pumps (3, 6). Among gram-negative bacteria, many of these MDR efflux pumps belong to the RND (resistance-nodulation-cell division) type family of tripartite efflux pumps. MDR in selected gram-negative bacteria has been shown to be reversible by using compounds like Phe-Arg-β-naphthylamide (PAβN) or other small N-heterocyclic organic compounds thought to inhibit RND type efflux pump activity through unknown mechanisms (4, 5, 7, 9). In this study, we describe another novel type of putative efflux pump inhibitor (EPI) identified through screening of an N-heterocyclic organic compound library for MDR reversal activity in *Escherichia coli*.

Selected arylpiperazines were synthesized at Steinbeis Transferzentrum Technische Chemie (Reutlingen, Germany) or purchased from Chess GmbH (Mannheim, Germany), Chem-Bridge Corporation (San Diego, Calif.), and Sigma Chemicals (St. Louis, Mo.). We initially screened the compound library through evaluating MICs of levofloxacin alone and in the presence of putative EPIs in *E. coli* test strains overexpressing *acrAB* and *acrEF* (see Table 1 for strain descriptions). Compounds were further tested if their minimal concentration required to reduce the levofloxacin MIC by fourfold (LVX-MRC₄) was at least fourfold lower than the intrinsic MIC of the test compound and if their MDR reversal effect (defined as reducing by at least fourfold the MICs of levofloxacin plus another antimicrobial agent) was observed in efflux pump-overexpressing test strains 2-DC14PS and 3-AG100MKX but not efflux pump-deficient control strains 1-DC14PS and HS276 (Table 1). MICs were determined by a standard microdilution assay using Luria-Bertani (LB) broth and a final inoculum of 5×10^5 CFU/ml.

Analysis of the relationship between the structure of phenylpiperazines and MDR reversal activity suggested that elongation of the spacer between the benzene ring and the piperazine

ring would enhance potency (Table 2). A fivefold increase in potency was observed for 1-(4-phenylbutyl)-piperazine (LVX-MRC₄, 100 μg/ml; spacer length, 4) compared to the weakest compound, 1-phenylpiperazine (PP) (LVX-MRC₄, >400 μg/ml; spacer length, 0). Halogenic substitutions at the benzene ring independently caused a significant increase in potency, with the most successful being the introduction of a trifluoromethyl group at the *meta* position of the benzene ring of PP. This compound, called mTFMPP (LVX-MRC₄, 50 μg/ml), became the most effective phenylpiperazine tested in our assay, whereas the naphthylpiperazines 1-(1-naphthyl)piperazine, 1-(1-naphthylmethyl)-piperazine (NMP), and 1-[4-(2-naphthyl)butyl]-piperazine were among the most potent unsubstituted arylpiperazines. Interestingly, the simple addition of an ethyl or phenyl group to the piperazine ring, as in 1-ethyl-4-(1-naphthyl)-piperazine or 1-phenyl-4-(1-naphthyl)-piperazine, led to a dramatic loss in potency.

NMP and mTFMPP had similar intrinsic MICs (NMP, 400 μg/ml; mTFMPP, 800 μg/ml) in efflux pump-overexpressing as well as efflux pump-deficient test strains and MDR reversal activities at the LVX-MRC₄ were limited to efflux pump-overexpressing test strains. Both compounds were compared with

TABLE 1. *E. coli* strains used in this study

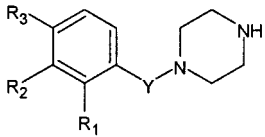
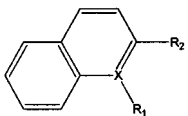
Strain	Description	Reference
3-AG100MKX ^a	<i>acrAB</i> -overexpressing <i>gyrA</i> mutant derived from AG100MKX (AG100 <i>marA</i> ::Km ^r)	2
DC14	AG100 <i>ΔacrAB</i> ::Km ^r	1
1-DC14PS	<i>gyrA</i> mutant derived from DC14PS after selection with ofloxacin	1
2-DC14PS ^b	<i>acrEF</i> -overexpressing mutant derived from 1-DC14PS after selection with ofloxacin	1
HS414	Wild-type W3110	8
HS276	HS414 <i>ΔacrEF ΔacrAB ΔyhiUV ΔacrD ΔyegMNO</i> ::Km ^r	8

^a Enhanced expression of *acrAB* without expression of *acrEF* was shown by reverse transcription-PCR and was associated with diminished intracellular accumulation of ofloxacin in the absence of CCCP (2).

^b Enhanced expression of *acrEF* was shown by reverse transcription-PCR and was associated with diminished intracellular accumulation of ofloxacin in the absence of CCCP (2).

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TABLE 2. Structure and activity as MDR reversal agents of selected phenyl- and arylpiperazines tested with *acrAB*-overexpressing *E. coli* 3-AG100MKX cells

Phenylpiperazine or arylpiperazine	Abbreviation	Y	X	R ₁	R ₂	R ₃	LVX-MRC ₄ (μg/ml)
Phenylpiperazine							
							
1-phenylpiperazine	PP	C ₀ H ₀	H	H	H	H	>400
1-(3-chlorophenyl)-piperazine	mCPP	C ₀ H ₀	H	Cl	H	H	100
1-(2-trifluoromethylphenyl)-piperazine	oTFMPP	C ₀ H ₀	CF ₃	H	H	H	100
1-(3-trifluoromethylphenyl)-piperazine	mTFMPP	C ₀ H ₀	H	CF ₃	H	H	50
1-(4-trifluoromethylphenyl)-piperazine	pTFMPP	C ₀ H ₀	H	H	H	CF ₃	50
1-benzylpiperazine	BP	C ₁ H ₂	H	H	H	H	250
1-(4-chlorobenzyl)-piperazine	pCBP	C ₁ H ₂	H	H	H	Cl	100
1-[3-(trifluoromethyl)benzyl]-piperazine	mTFMBP	C ₁ H ₂	H	CF ₃	H	H	200
1-(2-phenylethyl)-piperazine	PEP	C ₂ H ₄	H	H	H	H	>200
1-(3-phenylpropyl)-piperazine	PPP	C ₃ H ₆	H	H	H	H	200
1-(4-phenylbutyl)-piperazine	PBP	C ₄ H ₈	H	H	H	H	100
Arylpiperazine							
							
1-(1-naphthyl)piperazine	NP		C	pip ^a	H		50
1-(1-naphthylmethyl)-piperazine	NMP		C	CH ₂ -pip	H		50
1-[4-(1-naphthyl)butyl]-piperazine	1-NBP		C	C ₄ H ₈ -pip	H		50
1-[4-(1-naphthyl)butanoyl]-piperazine	1-NBOP		C	C ₃ H ₆ CO-pip	H		50
1-[4-(2-naphthyl)butyl]-piperazine	2-NBP		C	H	C ₄ H ₈ -pip		25
1-[4-(2-naphthyl)butanoyl]-piperazine	2-NBOP		C	H	C ₃ H ₆ CO-pip		50
1-ethyl-4-(1-naphthyl)-piperazine	ENP		C	pip-C ₂ H ₅	H		400
1-phenyl-4-(1-naphthyl)-piperazine	PNP		C	pip-C ₆ H ₅	H		400
quipazine	QP		N	H	pip		100

^a pip, piperazine.

PAβN in their ability to increase the intracellular accumulation of levofloxacin, measured as described previously (2). Briefly, levofloxacin (final concentration of 10 μg/ml) was added to *E. coli* 3-AG100MKX cells suspended to an optical density at 600 nm (OD₆₀₀) of 1 in 7 ml of 50 mM sodium phosphate buffer (pH 7.0) containing 0.2% glucose. After incubation at 37°C for 10 min, NMP or mTFMPP (each at a final concentration of 100 μg/ml), PAβN (final concentration of 25 μg/ml), or carbonyl cyanide *m*-chlorophenyl-hydrazone (CCCP) (200 μM) were added. PAβN and CCCP were from Sigma Chemicals. The concentration of PAβN chosen represented the LVX-MRC₄ of PAβN for strain 3-AG100MKX. At timed intervals, 1-ml samples were removed and centrifuged through silicone oil, and the pellet was resuspended in 300 μl of 0.1 M glycine hydrochloride (pH 3.0). After overnight incubation at room temperature, samples were again centrifuged, and the amount of released levofloxacin was determined spectrofluorometrically (excitation, 292 nm; emission, 496 nm) in the supernatant. Experiments were done in triplicate.

As shown in Fig. 1, addition of both NMP and PAβN but not of mTFMPP resulted in increased intracellular levofloxacin accumulation, consistent with an EPI effect. To further confirm this finding, we measured intracellular ethidium bromide

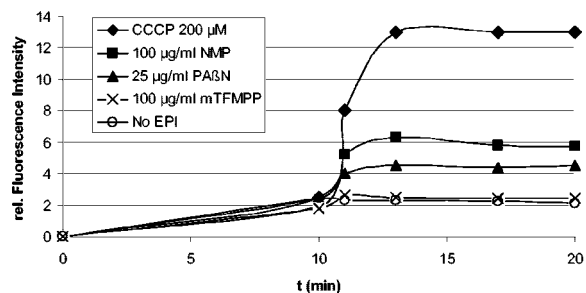


FIG. 1. Intracellular accumulation of levofloxacin with or without addition of NMP, mTFMPP, PAβN, and CCCP in the *acrAB*-overexpressing *E. coli* strain 3-AG100MKX. Levofloxacin (10 μg/ml) was added at time point 0, and NMP, mTFMPP, PAβN, or CCCP were added after 10 min. Samples removed at timed intervals were centrifuged through silicone oil, and the pellet was resuspended in 0.1 M glycine hydrochloride (pH 3.0). After overnight incubation at room temperature, samples were again centrifuged, and the amount of released levofloxacin in the supernatant was determined spectrofluorometrically. Shown are representative results from one out of three experiments.

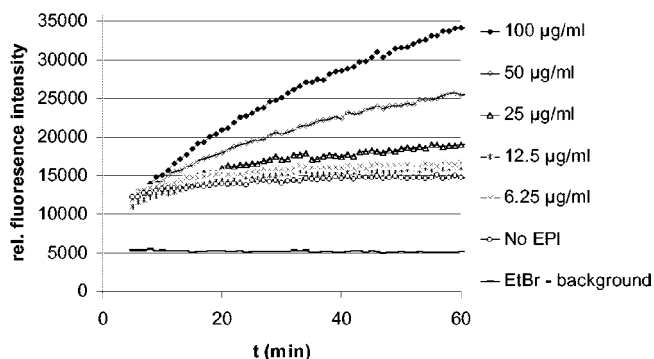


FIG. 2. Effect of the addition of increasing concentrations of NMP on EtBr accumulation in *acrAB*-overexpressing *E. coli* strain 3-AG100MKX. Cells grown in LB agar overnight were suspended in phosphate-buffered saline containing 0.4% glucose, and NMP was added at increasing concentrations. EtBr was added (1 $\mu\text{g/ml}$) at time point 0, and fluorescence changes over time were measured by fluorescence spectroscopy.

(EtBr). 3-AG100MKX cells were grown overnight on LB agar plates and suspended in phosphate-buffered saline plus 0.4% glucose (pH 7.4) to an OD_{600} of 1, and NMP was added at increasing concentrations. Samples were placed into a 96-well plate, EtBr was added (final concentration, 1.0 $\mu\text{g/ml}$), and fluorescence was read in a Safire (Tecan, Crailsheim, Germany) fluorescence plate reader (excitation, 518 nm; emission, 605 nm). Figure 2 shows that addition of NMP resulted in a dose-dependent increase in relative fluorescence intensity. Similar results were obtained with 2-DC14PS and HS414 but not with 1-DC14PS and HS276, in which the effects were much smaller (data not shown). These observations suggested that NMP may exert its MDR reversal activity primarily through inhibition of the AcrAB and AcrEF efflux pumps.

The spectrum of antimicrobial agents affected by addition of NMP (at a concentration of 100 $\mu\text{g/ml}$) and, for comparison, of PA β N (at a concentration of 25 $\mu\text{g/ml}$, corresponding to one-fourth the MIC for pump-deficient strain 1-DC14PS) was determined by evaluating the reduction of MICs of several agents. As shown in Table 3, NMP reduced the MIC of levofloxacin by 8- to 16-fold in *E. coli* strains overexpressing *acrAB* or *acrEF*. Effects on other agents were similar or slightly smaller, with 4- to 8-fold reductions of the MICs of oxacillin, rifampin, chloramphenicol, and clarithromycin. The MIC of linezolid was differently affected in the *acrEF*-overexpressing strain 2-DC14PS. The reduction was 32-fold for this strain versus 8-fold for the *acrAB*-overexpressing strain 3-AG100MKX (Table 3). The results obtained with *E. coli* strain HS414 and its pump-deficient derivative HS276 were similar (data not shown) to those obtained with 1-DC14PS and 2-DC14PS (Table 3). Generally, the MICs for pump-deficient strains 1-DC14PS and HS276 were not affected by addition of NMP, with the exception of rifampin, while PA β N addition to the pump-deficient mutant strains led to decreased MICs of agents other than chloramphenicol. Addition of PA β N at 25 $\mu\text{g/ml}$ in efflux pump-overexpressing strains appeared to be more effective than addition of NMP, in particular regarding clarithromycin but not levofloxacin, linezolid, and tetracy-

cline susceptibilities (Table 3). This finding did not change after increasing the concentration of PA β N to 100 $\mu\text{g/ml}$ (data not shown).

Additional representatives of diverse classes of agents were tested with 3-AG100MKX. A MIC reduction (fourfold or greater) after addition of NMP was observed for other fluoroquinolones (ciprofloxacin, norfloxacin, enoxacin, and pefloxacin), erythromycin, azithromycin, clindamycin, doxycycline, nitrofurantoin, and EtBr but not for the ketolides telithromycin and ABT-773, glycopeptides, aminoglycosides, trimethoprim-sulfamethoxazole, and fosfomycin (data not shown).

Among the tested arylpiperazines, the naphthyl derivative NMP matched our predefined working criteria of an ideal EPI: it significantly reduced the MICs of two or more antibiotics in efflux pump-overexpressing strains, it did not inhibit the growth of an efflux pump-deficient strain at concentrations effective in efflux-competent strains, and finally, it increased the intracellular accumulation of otherwise expelled efflux pump substrates. NMP, as the most potent compound in *E. coli*, appeared to be less active than PA β N, which, unlike NMP, showed inhibitory activity in strains without expression of the AcrAB and AcrEF *E. coli* efflux pumps and thus may exert its effect through action on other pumps or through additional mechanisms unrelated to pump inhibition.

NMP can be added to the list of MDR reversal agents with putative EPI activity in *E. coli*. Expanding this list to include compounds with different spectra of activity in terms of bacterial species as well as drugs may help with investigation in more detail of the mechanisms of pump substrate recognition and mechanisms of MDR efflux pump inhibition.

TABLE 3. Effect of the putative EPI NMP and of PA β N on MICs of different drugs in efflux pump-overexpressing *E. coli* strains 2-DC14PS and 3-AG100MKX and ΔacrAB control strain 1-DC14PS

Strain	Drug	MIC ($\mu\text{g/ml}$) with:		
		No EPI	NMP (100 $\mu\text{g/ml}$)	PA β N (25 $\mu\text{g/ml}$)
1-DC14PS	Levofloxacin	0.125	0.125	0.06
	Linezolid	16	16	8
	Clarithromycin	2	2	1
	Oxacillin	≤ 0.5	≤ 0.5	≤ 0.5
	Rifampin	8	2	≤ 0.06
	Chloramphenicol	1	1	1
	Tetracycline	1	0.5	0.25
2-DC14PS	Levofloxacin	4	0.25	1
	Linezolid	512	16	64
	Clarithromycin	256	32	8
	Oxacillin	64	16	32
	Rifampin	16	4	0.25
	Chloramphenicol	8	1	1
	Tetracycline	2	0.5	2
3-AG100MKX	Levofloxacin	4	0.5	0.5
	Linezolid	256	16	64
	Clarithromycin	256	32	2
	Oxacillin	64	16	32
	Rifampin	16	4	0.125
	Chloramphenicol	8	1	2
	Tetracycline	2	0.5	2

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