

# In vitro Enhancement of Gram-positive Antibacterial Compounds by Recently Described Efflux Pump Inhibitors (EPIs) in MDR *Escherichia coli* and Comparison with the Membrane Permeabilizer Polymyxin B nonapeptide (PMBN)

Tobias Schweigger, Martina Vavra, Ludwig Greim-Kuczewski, Nicole Specht, Sabine Schuster, and Winfried V. Kern

Division of Infectious Diseases, University Hospital & Medical Center, Freiburg, Germany

## Objectives

Bacterial multidrug resistance (MDR) is increasing among potentially pathogenic gram-negative bacteria (GNB) and is often associated with reduced membrane permeability or enhanced efflux. EPIs or membrane permeabilizers (MPs) can sensitize GNB and enhance the activity of a variety of anti-gram-positive compounds.

We evaluated the sensitizing effects of new putative EPIs including

- MBX2319 (a novel pyranopyridine EPI; Oppermann et al AAC 2014) and
- various phytochemicals thought to interact with AcrB residues in close proximity to binding site 1 according to modeling studies (Aparna et al PLoS ONE 2014).

## Methods

Various *E. coli* reference, laboratory, and clinical isolates were used. Test strains included 33 clinical isolates from various parts of the world that showed multidrug resistance (to third-generation cephalosporins, fluoroquinolones, trimethoprim-sulfamethoxazole and tetracycline) and had different levels of *acrB* and *OmpC/OmpF* expression. All test strains showed  $\geq 4$ -fold reduction of linezolid (up to 32-fold) and tetracycline (up to 16-fold) MICs with NMP (Table).

MBX2319 was from T.J. Oppermann (Microbiotix, Worcester, Mass./USA), and the phytochemicals were from Sigma. They were used at subinhibitory concentrations. Control EPIs were NMP (100  $\mu\text{g}/\text{mL}$ ) and PA $\beta$ N (25  $\mu\text{g}/\text{mL}$ ).

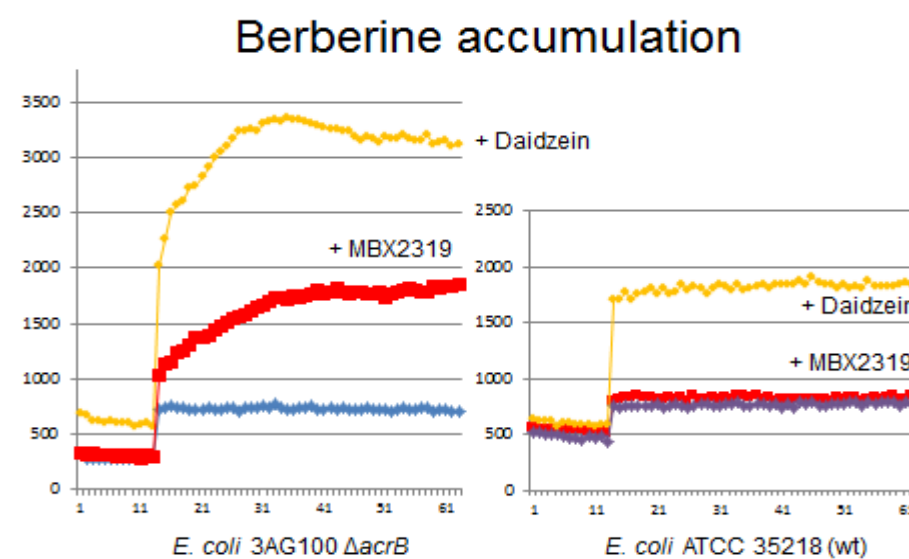
Drug	MIC <sub>50</sub> ( $\mu\text{g}/\text{mL}$ )	No. of isolates (%) with indicated fold reduction of MIC after addition of							
		MBX 2319 25 $\mu\text{M}$		NMP 100 $\mu\text{g}/\text{mL}$		PA $\beta$ N 25 $\mu\text{g}/\text{mL}$		PMBN 1 $\mu\text{g}/\text{mL}$	
		$\geq 4$ -fold	$\geq 16$ -fold	$\geq 4$ -fold	$\geq 16$ -fold	$\geq 4$ -fold	$\geq 16$ -fold	$\geq 4$ -fold	$\geq 16$ -fold
Levofloxacin	8	4	-	14	1	12	1	2	1
Moxifloxacin	16	1	-	23 (70%)	-	32 (97%)	2	3	-
Tetracycline	128	8	-	33 (100%)	2	21 (64%)	-	22 (67%)	-
Minocycline	8	16	-	31 (94%)	20 (61%)	28 (85%)	12	23 (70%)	2
Tigecycline	0,5	1	-	10	-	-	-	-	-
Oxacillin	>1024	-	-	3	-	9	-	7	-
Chloramphenicol	8	2	-	23 (70%)	1	16	-	17 (52%)	-
Linezolid	256	12	-	33 (100%)	14	31 (94%)	2	30 (91%)	6
Rifampicin	8	-	-	26 (79%)	1	32 (97%)	32 (97%)	31 (94%)	28 (85%)
Rifaximine	8	1	-	18 (55%)	-	32 (97%)	32 (97%)	31 (94%)	29 (88%)
Clindamycin	64	5	-	27 (82%)	3	31 (94%)	8	32 (97%)	11
Erythromycin	1024	-	-	16	-	33 (100%)	32 (97%)	33 (100%)	29 (88%)
Clarithromycin	128	-	-	10	1	33 (100%)	33 (100%)	33 (100%)	32 (97%)
Azithromycin	32	-	-	28 (85%)	3	32 (97%)	26 (79%)	32 (97%)	14
Novobiocin	32	5	-	19 (58%)	-	29 (88%)	18 (55%)	33 (100%)	27 (82%)
Gentamicin *	32	-	-	-	-	-	-	-	-
Streptomycin *	64	-	-	-	-	-	-	-	-

\* used as negative (usually non-effluxed) control drugs

PMBN, at 1  $\mu\text{g}/\text{mL}$  was used as MP. Synergistic activity was defined as  $\geq 4$ -fold MIC reduction. Tests were done at least in duplicate, using MHB, LB, RPMI or MOPS.

Dye accumulation was determined in the absence and presence of EPIs. Dye was added after 10 minutes of incubation with EPI at 34°C with shaking, and fluorescence was measured at 34°C over time with a fluorescence plate reader (Tecan SAFIRE).

Dyes were ethidium bromide (final concentration 25  $\mu\text{M}$ , excitation 518 nm; emission 605 nm), Hoechst 33342 (2.5  $\mu\text{M}$ , excitation 350 nm; emission 461 nm), berberine (30  $\mu\text{g}/\text{mL}$ , excitation 355 nm; emission 517 nm), and PA $\beta$ N (added at 200  $\mu\text{M}$ , estimated by measuring  $\beta$ -naphthylamine, excitation 320 nm; emission 460 nm).



## Results

MBX2319 was used at a final concentration of 25  $\mu\text{M}$  at which there was no bacterial growth inhibition. Higher concentrations could not be tested due to solubility problems.

At this concentration the compound was synergistic (up to 8-fold MIC reduction) with minocycline in 16 clinical isolates, with linezolid in 12 isolates, and with tetracycline in 8 isolates (Table).

The synergy tests with other drugs generally yielded poorer results. MBX2319 was inactive in  $\Delta toI/C$  and  $\Delta acrB$  test strains.

In fluorescent dye accumulation tests (different dyes) with reference and clinical strains MBX2319 did not enhance accumulation (Figure, showing results for *E. coli* ATCC35218 with berberine).

The following phytochemicals were initially tested in synergy tests with and without addition of Mg:

- lanatoside C (a cardiac glycoside)
- umbelliferone (a coumarin derivative),
- daidzein (an isoflavone)
- gentisic acid (2,5-dihydroxybenzoic acid),
- protocatechuic acid (3,4-dihydroxybenzoic acid).

None of them showed synergy with any of the test drugs (including levofloxacin). Accumulation of some dyes was enhanced in both *acrB* wildtype as well as in  $\Delta acrB$  test strains (Figure, showing results for *E. coli* ATCC35218 and a knockout strain with daidzein and berberine).

PMBN reduced the test drug MICs  $\geq 4$ -fold in >90% of the strains for all macrolides/clindamycin and novobiocin, rifampicin/rifaximine, and linezolid. PMBN activity correlated well with PA $\beta$ N (which has known MB effects) but not with NMP or MBX2319 activity.

## Conclusion

In our hands, the recently described EPIs showed limited or no activity in MDR *E. coli* clinical isolates. Screening for more potent EPIs is urgently needed.