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Role of MexAB-OprM in intrinsic resistance of *Pseudomonas aeruginosa* to temocillin and impact on the susceptibility of strains isolated from patients suffering from cystic fibrosis

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Sir,

Temocillin (6-α-methoxy-ticarcillin) is resistant to most β-lactamases, including AmpC and extended-spectrum β-lactamases, and is therefore considered a useful alternative to carbapenems in infections caused by several resistant Gram-negative pathogens.[1] Yet, temocillin is inactive against

Table 1. MICs of temocillin and ticarcillin for *P. aeruginosa* strains with known efflux characteristics, as measured in Mueller–Hinton broth (MHB) or in MHB supplemented with the broad-spectrum efflux inhibitor PAβN (50 mg/L)

Strain	Origin or ref.	Description	Efflux characteristics, gene expression level					MIC (mg/L)	
			mexA[a]	*mexX*[a]	*oprM*[a]	*mexC*[b]	*mexE*[b]	temocillin (+PAβN[c])	ticarcillin (+PAβN[c])
Reference strain									
PAO1	ATCC		1	1	1	—	—	256–512 (64)	32 (16)
Engineered strains									
FB1	[d]	PAO1 *mexB::FRT*	ND	ND	ND	ND	ND	2	0.5
CB536	[e]	PAO1 Δ*mexCD-oprJ*	1.09	1.65	ND	−	+	128 (16)	8 (1)
CB603	[e]	PAO1 Δ*mexEF-oprN*	1.21	1.02	0.51	−	−	128 (32)	16 (16)
CB602	[e]	PAO1 *mexXY::FRT*	1.10	*0.06*	0.55	−	+	64 (16)	16 (16)
PAO1 mexAB	[f]	PAO1 *mexAB::FRT*	*0*[g]	1.08	ND	−	+	4 (2)	2 (2)
PAO200	[f]	PAO1 Δ*mexAB-oprM*	*0*[g]	1.26	ND	−	−	4 (0.5)	2 (0.5)
SG01	[h]	PAO1 Δ*oprM*	ND	ND	ND	ND	ND	2	0.5
CMZ091	[i]	PAO1 Δ*mexZ* (MeXY overproducer)	ND	ND	ND	ND	ND	256	16
CM114	[h]	PAO1 Δ*mexXY*	ND	ND	ND	ND	ND	256	32
4098	[j]	PAO1 *met-9020 pro-9024 blaP-9208* (weak AmpC producer)	1.26	1.62	0.33	−	−	128	8
4098E	[k]	4098 overproducing MexAB-OprM	**5.41**	1.31	**3.19**	−	−	1024 (512)	64 (32)
4098ET	[k]	4098E Δ*oprM*	**2.18**	*0.04*	*0.02*	−	−	2 ([l])	2 ([l])
PA Δ*dacB*	[m]	PAO1 Δ*dacB::lox* (AmpC overproducer)	ND	ND	ND	ND	ND	128	64
Clinical isolates from patients with HAP									
168B	[n]		1.15	0.89	ND	−	−	256 (32)	16 (16)
156	[n]		0.33	0.95	ND	−	+	512 (64)	256 (32)
68	[n]		0.87	**44.94**	ND	−	−	512 (64)	32 (16)
34	[n]		**6.86**	1.26	ND	−	−	>1024 (512)	256 (128)
333A	[n]		**2.17**	2.29	ND	−	−	>1024 (1024)	128 (128)
11	[n]		**3.56**	**5.68**	ND	−	−	1024 (64)	32 (32)
12	[n]		**3.97**	**9.04**	ND	+	+	512 (128)	64 (64)

			Efflux characteristics, alterations					
			mexA	MexA	*mexB*	MexB		
Clinical isolates from cystic fibrosis patients								
3020S	[d]		—	—	—	—	128	16
3020R	[d]	isogenic to 3020S with deletion in *mexA*	Δ 112 nt (370–482)	aberrant	—	—	2	1
3525			—	—	—	—	512	32
3807		isogenic to 3525 with mutation in *mexA*	G214A	G72S	—	—	32	4
2715	[d]	mutation in *mexA*	A590G	Y197C	—	—	32	2
616		mutation in *mexA*	C752T	S251F	—	—	1	0.5
2729	[d]	deletion in *mexA*	Δ 8 nt (576–583)	aberrant	—	—	2	1
2933	[d]	deletion in *mexA*	Δ 1 nt (870)	aberrant	—	—	2	0.5
2998	[d]	deletion in *mexA*	C205T	truncated	—	—	2	0.25
2721	[d]	deletion in *mexA*	Δ 1 nt (860)	aberrant	—	—	1	0.25
2716	[d]	mutation in *mexB*	—	—	A776T	Q259L	1	0.5
2804	[d]	deletion in *mexB*	—	—	Δ 1 nt (2147)	aberrant	4	1
2858	[d]	deletion in *mexB*	—	—	Δ 1 nt (494)	aberrant	1	0.5
3066		deletion in *mexB*	—	—	G2364A	truncated	1	0.5

ND, not determined.
[a]Real-time quantitative PCR [threshold ratio compared with PAO1; values shown in bold are considered as denoting highly significant overexpression (≥2 and ≥5 for *mexA* and *mexX*, respectively, based on the recommendations of the manufacturer of the kit used for their detection; no threshold value set for *oprM*); values interpreted as denoting an absence (or quasi-absence) of detection are shown in italics].
[b]RT–PCR [semi-quantitative detection (+/−)].
[c]PAβN (broad-spectrum efflux inhibitor) used at 50 mg/L.
[d]Vettoretti *et al. Antimicrob Agents Chemother* 2009; **53**: 1987–97.
[e]Robertson *et al. J Bacteriol* 2007; **189**: 6870–81.
[f]Mima *et al. J Bacteriol* 2007; **189**: 7600–9.
[g]Complete absence of detection.
[h]S. Guénard and P. Plésiat (unpublished results).
[i]Muller *et al. Antimicrob Agents Chemother* 2011; **55**: 1211–21.
[j]Li *et al. Antimicrob Agents Chemother* 1994; **38**: 1732–41.
[k]Hamzehpour *et al. Antimicrob Agents Chemother* 1995; **39**: 2392–6.
[l]No growth in the presence of PAβN (PAβN MIC = 25 mg/L for this strain).
[m]Moya *et al. PLoS Pathog* 2009; **5**: e1000353.
[n]Isolated from ICUs in Belgium.

Pseudomonas aeruginosa, possibly because of poor permeation across the outer membrane barrier and/or reduced binding to penicillin-binding proteins.[1] However, the role of multidrug efflux systems has not been examined so far. Three multidrug efflux systems have been reported to export β-lactams in *P. aeruginosa*, namely (from least to most effective) MexXY-OprM, MexCD-OprJ and MexAB-OprM.[2] We wondered whether temocillin could be the substrate of one or several of these transporters.

Temocillin (Eumedica, Brussels, Belgium) and ticarcillin (disodium salt; Sigma–Aldrich, St Louis, MO, USA) were tested against: (i) the wild-type reference strain PAO1; (ii) a panel of laboratory strains with specific disruption(s) of the gene(s) encoding the three transporters mentioned above and MexEF-OprN, another efflux pump accommodating fluoroquinolones, trimethoprim and chloramphenicol, but not β-lactams,[2] and producing different levels of AmpC; (iii) clinical isolates from patients hospitalized in intensive care units (ICUs) with hospital-acquired pneumonia (HAP); and (iv) strains from cystic fibrosis patients that were found to be hypersusceptible to carbenicillin and ticarcillin (Tic^{HS} phenotype) due to mutations in *mexA* or *mexB*.[3] MICs were determined by microdilution in Mueller–Hinton broth (pH 7.4, 24 h) without or with the broad-spectrum efflux inhibitor Phe-Arg-β-naphthylamide (PAβN; 50 mg/L; Sigma–Aldrich).[4] The expression of *mexA* and *mexX* was measured by quantitative real-time PCR, and that of *mexC* and *mexE* was measured by semi-quantitative RT–PCR. *mex*A, *mexB* and *oprM* were sequenced in strains from cystic fibrosis patients.[3]

The MIC of temocillin for PAO1 was ≥256 mg/L, but fell to 64 mg/L when tested in the presence of PAβN, a broad-spectrum competitive inhibitor of efflux transporters (Table 1), suggesting a role of active efflux in the intrinsic high-level resistance of *P. aeruginosa* to temocillin. The magnitude of the inhibitory effect of PAβN, however, varies depending on the substrate.[4] To better quantify the impact of efflux on temocillin MICs, and also to identify the transporter(s) responsible for its efflux, we used isogenic strains deficient in the main efflux systems. Disruption of MexCD-OprJ, MexEF-OprN or MexXY only slightly affected the temocillin MIC (2–3 $\log_2$ reduction), consistent with the strongly repressed expression of these three systems in wild-type strains. In contrast, disruption of *mexB*, *mexAB*, *oprM* or *mexAB-oprM* decreased MICs to values as low as 2–4 mg/L, with a minimal additional effect of PAβN. Conversely, overexpression of *mexAB*, but not of *mexXY*, further increased the temocillin MIC compared with PAO1. This clearly indicates that MexAB-OprM-driven efflux strongly contributes to the intrinsic resistance of *P. aeruginosa* to temocillin, while the other Mex systems only play a minor role. We also confirmed the stability of temocillin to AmpC.

To examine the clinical relevance of our observations, we measured temocillin MICs for isolates collected from ICU patients with HAP. All values were high, but those from isolates overexpressing *mexA* were higher than those for PAO1, corroborating the importance of this efflux system in temocillin resistance. In parallel, we found that isolates obtained from cystic fibrosis patients and showing hypersusceptibility to ticarcillin were also hypersusceptible to temocillin, with MICs ranging between 1 and 4 mg/L in most cases. Interestingly enough, however, the MICs for some isolates with single nucleotide mutations in MexA (G72S and Y197C) remained moderately elevated (32 mg/L), suggesting that these mutated proteins remained partly functional.

Noteworthy, when considering all isolates examined here, differences between temocillin and ticarcillin MICs were much greater in isolates producing a functional or partially functional MexAB-OprM pump than in deficient strains (with temocillin MICs being 3–5 $\log_2$ dilutions higher than those of ticarcillin). This suggests that temocillin is a preferential substrate for the MexAB-OprM transporter compared with ticarcillin, pointing to a potential role of the 6-α-methoxy substituent in its recognition and efflux.

While intrinsic resistance of *P. aeruginosa* to temocillin makes this antibiotic unusable in most conventional clinical set-ups, we see here that impairment of efflux lowers the MICs to values below the current clinical susceptibility breakpoint for Enterobacteriaceae (16 mg/L; UK and Belgium) or even the pharmacokinetic/pharmacodynamic breakpoint proposed for a 4 g daily dose (8 mg/L).[5] This may further trigger current efforts in designing clinically useful inhibitors of the MexAB-OprM transporter, since such combined therapy could provide the clinician with a useful alternative to current antipseudomonal β-lactams, especially if considering temocillin's remarkable β-lactamase stability. The present data may also have potential immediate application for cystic fibrosis patients. These patients can be infected by *Burkholderia cepacia*, against which temocillin is active and, therefore, commonly used.[1] Because of the large prevalence of *P. aeruginosa* isolates with the hypersusceptible Tic^{HS} phenotype in this patient population,[3] temocillin could contribute to their eradication as well. Testing for temocillin susceptibility of *P. aeruginosa* isolated from cystic fibrosis patients appears, therefore, potentially useful.

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Transparency declarations

P. M. T. is an unpaid adviser of Eumedica (manufacturer of temocillin); he does not have any financial interests in this company. J. M. B., S. G., P. P. and F. V. B. have no conflicts of interest to declare.

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