

In-vivo impact of the MexXY efflux system on aminoglycoside efficacy in an experimental model of *Pseudomonas aeruginosa* pneumonia treated with tobramycin

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ABSTRACT

Aminoglycosides are of major importance in treating *Pseudomonas aeruginosa* pneumonia (PAP). However, their efficacy may be compromised by low-level resistance caused by the inducible MexXY multidrug efflux pump. In the present study, the impact of the MexXY efflux pump was investigated *in vivo* in an experimental model of PAP in rabbits treated with intravenous tobramycin. Three strains were used to induce PAP in rabbits: PAO1 (wild-type strain; MIC 1 mg/L), mutant 11B (*mexX::Tn501*; no expression of MexXY; MIC 0.5 mg/L) and mutant MutGR1 (MexZ null; constitutive expression of MexXY; MIC 2 mg/L). Five hours after inoculation, treatment with tobramycin (10 mg/kg) was implemented (peak serum concentration 30 mg/L). The animals were killed humanely 48 h after inoculation, and the residual pulmonary bacterial concentration was determined. Selection of bacteria expressing MexXY was determined by plating lung homogenates on agar plates containing antibiotic. Mean bacterial counts (\log_{10} CFU/g) for treated vs. untreated rabbits were 6.26 and 8.13 ($p < 0.0001$), 6.00 and 8.38 ($p < 0.001$), and 7.25 and 8.79 ($p 0.04$) for PAO1, 11B and MutGR1, respectively, with an overall mortality rate of 0% vs. 8.9% ($p < 0.01$). MexXY-overexpressing bacteria were recovered from three (21%) treated rabbits. The C_{\max} /MIC ratio was the parameter that was best associated with tobramycin efficacy. The bacteria overexpressing MexXY, recovered from lung, occurred with a C_{\max} /MIC window of 19–26. It was concluded that the experimental PAP model highlights poor tobramycin bacteriological efficacy *in vivo*, contrasting with survival gain, and that the contribution of the MexXY system to this low level of tobramycin efficacy is modest. Finally, this model appears to be suitable for the investigation of new anti-pseudomonal therapeutic strategies.

Keywords Efflux, MexXY, pneumonia, *Pseudomonas aeruginosa*, rabbit model, tobramycin

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INTRODUCTION

While aminoglycosides are the cornerstone antibiotics for the treatment of *Pseudomonas aeruginosa* lung infections, either for ventilator-associated pneumonia or for patients with cystic fibrosis, their clinical efficacy may be compromised *in vivo*, even though *in-vitro* assays predict good antimicrobial activity [1]. Among known mechanisms of resistance in *P. aeruginosa*, the polyspecific efflux system MexXY (also known as AmrAB), which

belongs to the RND (resistance/nodulation/cell division) family of transporters, is thought to play a key role in the resistance of *P. aeruginosa* to aminoglycosides [2]. Expression of the *mexXY* operon is regulated negatively by the product of the upstream putative repressor gene *mexZ* [2–4]. Overproduction of the MexXY efflux pump is inducible, in part, by its substrate antibiotics, including aminoglycosides, macrolides and tetracyclines (but not fluoroquinolones, chloramphenicol or β -lactams), thereby allowing *P. aeruginosa* to adapt rapidly to inhibitory concentrations of these antibiotics [5,6]. This so-called adaptive resistance [7,8], generally considered to be low-level, disappears when the organism is no longer in contact with these antibiotics. MexXY proteins

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may also be overproduced constitutively as a result of mutations occurring inside or outside the *mexZ* gene, or inside unknown additional genes involved in the regulation of MexXY expression [2,4]. Overexpression of the MexXY system in strain PAO1 (either by adaptive resistance or following a mutational event) leads to a two- to eight-fold increase in in-vitro MICs of aminoglycosides and fluoroquinolones compared with those for the wild-type parental strain.

While data are accumulating on the occurrence of MexXY-overproducing strains in the clinical setting [2,9,10], little is known about the possible implications of this system for the emergence of isolates with reduced susceptibility to aminoglycosides, and the resulting consequences for the efficacy of aminoglycoside treatment. In the present study, MexXY overproduction was explored in an experimental model of *P. aeruginosa* pneumonia in rabbits, which then received treatment with intravenous tobramycin in a regimen similar to that used to treat humans. The impact of overexpression of the MexXY system on tobramycin efficacy in this pneumonia model was also evaluated.

MATERIALS AND METHODS

Bacterial strains, growth conditions and antibiotics

P. aeruginosa PAO1 was used as the wild-type reference strain. Mutant 11B is a *mexX::Tn501* insertion derivative of PAO1 showing hyper-susceptibility to aminoglycosides, erythromycin and tetracyclines [3]. Mutant MutGR1 is another derivative of PAO1, and hyper-produces protein MexY constitutively as a result of a nucleotide substitution (C→T) at position 307 in the repressor gene *mexZ* [2]. Bacteria were cultured at 37°C, either in Mueller–Hinton broth with adjusted concentrations of Ca²⁺ and Mg²⁺ (MHB; Difco Laboratories, Elancourt, France) or on Mueller–Hinton agar plates (MHA; bioMérieux, Marcy l’Etoile, France). When necessary, selective growth media were made by addition of both gentamicin and ciprofloxacin, at concentrations of 2 mg/L and 0.125 mg/L, respectively.

In-vitro susceptibility testing

Drug MICs were determined by the standard agar dilution method recommended by the Comité de l’Antibiogramme de la Société Française de Microbiologie [11]. The antibiogram of wild-type strain PAO1 displays apparent antagonism between ciprofloxacin and aminoglycoside disks, as a result of in-vitro adaptive resistance induced by the aminoglycoside, leading to decreased susceptibility to ciprofloxacin. The antibiogram of PAO1 overexpressing MexXY efflux is similar to that of strain MutGR1, with decreased susceptibility to all aminoglycosides, ciprofloxacin and cefepime, and

loss of antagonism between aminoglycosides and ciprofloxacin. The antibiogram of strain 11B shows increased susceptibility to aminoglycosides, ciprofloxacin and cefepime, and no antagonism between aminoglycosides and ciprofloxacin, because of the absence of an efficient MexXY system [2,3,12].

Preparation of the inoculum

Before each animal experiment, one aliquot of the selected strain was inoculated into MHB, cultured on agar plates, and then incubated for 24 h at 37°C. Three colonies were then taken, incubated in 10 mL of MHB for 6 h at 37°C, and then cultured on an agar plate for 18 h at 37°C. This culture was then diluted in physiological saline to obtain a final concentration of 9.5 log₁₀ CFU/mL. No adjuvant was used. These concentrations were determined initially by OD measurements with reference to a standard curve, and were then confirmed by using successive dilution cultures.

Experimental *P. aeruginosa* pneumonia in rabbits

Male New Zealand White immunocompetent rabbits (body weight 2.5–3.2 kg) were obtained from CEGAV (Saint Mars d’Egrenne, France), placed in individual cages, and provided with food and water *ad libitum*. Installation of central venous catheters and production of pneumonia were as described previously [13,14]. In brief, 24 h after jugular catheterisation, bacterial pneumonia was induced by endobronchial challenge with 0.5 mL of saline containing 9.5 log₁₀ CFU/mL. Treatment was by infusion, started 5 h after bacterial challenge, and lasted for 2 days. Tobramycin was delivered through the first central venous catheter, with changing infusion rates, delivered by a computer-controlled electric pump, in order to simulate the tobramycin kinetics observed in human serum following a dose of 10 mg/kg given intravenously once-daily (concentration at the end of the infusion, C_{max} 30 mg/L) [15,16].

Pharmacokinetic analysis

For each animal, antibiotic serum concentrations were determined on iterative blood samples, obtained through the second central catheter. Tobramycin concentrations were determined by a disk plate bioassay with antibiotic medium II (Difco) and *Bacillus subtilis* ATCC 9466 as the indicator organism (limit of detection 0.1 mg/L). Standard curves were established with antibiotic solutions (0.5–7 mg/L) in serum. The linearity of the standard curves used for disk plate bioassays was ≥0.97 (r²). Serum samples were diluted in serum where necessary to ensure that their concentrations were within the range of those on the standard curve. The standards were assayed for each experiment, and serum concentrations were assayed in duplicate. The between-day and within-day coefficients of variation for replicates were 10.7% and 11.7%, respectively. The level of plasma protein binding was considered to be null [17].

Evaluation of infection

The rabbits were anaesthetised and killed humanely 48 h after inoculation. Twenty-three animals were used as controls without treatment (13, six and four animals for strains PAO1,

11B and MutGR1, respectively), and 46 animals were treated with intravenous tobramycin (18, 11 and nine animals infected with strains PAO1, 11B and MutGR1, respectively). The spleen and both lungs of each rabbit were weighed and homogenised in sterile water. Bacteria were enumerated by serial dilution on MHA, followed by incubation of the plates for 24 h at 37°C. Bacterial concentrations in each lung and in the spleen were determined after adjusting for weight. For each rabbit, the mean pulmonary bacterial concentration was calculated using the bacterial concentration in each lung (expressed as CFU/g).

In the group of rabbits infected with strain PAO1, bacteria expressing MexXY efflux were detected by plating the crude homogenate tissue and ten-fold dilutions on MHA made selective by the addition of gentamicin and ciprofloxacin as described above, followed by incubation for 72 h at 37°C. An antibiogram was determined for any colonies recovered on this selective medium to determine whether the MexXY phenotype was present (see above). Colonies overexpressing MexXY efflux were then tested for reversion by performing a new antibiogram after seven subcultures on antibiotic-free MHA.

Pharmacodynamic analysis

The following parameters related to MIC were calculated from the individual pharmacokinetics of each treated animal: C_{\max}/MIC ; area under the curve of serum concentration vs. time (0–24 h) $\text{AUC}_{0-24}/\text{MIC}$; and time of concentration above MIC ($T > \text{MIC}$, expressed as a percentage). These parameters were termed pharmacodynamic–pharmacokinetic parameters.

Statistical analysis

Results were expressed as means \pm SD. Quantitative variables were compared to analysis of variance and eventually completed by a post-hoc analysis using the Bonferroni test. Proportions percentages were compared using the Fisher exact test. The quantitative relationship between antimicrobial efficacy and each of the pharmacodynamic–pharmacokinetic parameters was determined by using an E_{\max} model (Hill formula). Kaplan–Meier methodology was used for the survival study, using the log-rank test, with multivariate analysis performed with a Cox model. $p < 0.05$ was considered significant.

RESULTS AND DISCUSSION

Experimental *P. aeruginosa* pneumonia in rabbits

The MICs for the three strains used to infect the rabbits are summarised in Table 1. All strains were susceptible to tobramycin ($\text{MIC} \leq 4$ mg/L) and ciprofloxacin ($\text{MIC} \leq 1$ mg/L). Only strain MutGR1 was resistant to gentamicin ($\text{MIC} > 8$ mg/L). When the serum concentration vs. time curve of tobramycin observed in rabbits was superimposed on human pharmacokinetic data [16], there was no difference between the rabbit groups, as defined by strains, with respect to

Table 1. Strains of *Pseudomonas aeruginosa* used in this study

Strain	Relevant properties	MexXY efflux	MIC (mg/L)		
			Tobramycin	Gentamicin	Ciprofloxacin
PAO1	Wild-type	Inducible	1	4	0.125
11B	<i>mexX</i> null	Non-functional	0.5	0.5	0.125
MutGR1	<i>mexZ</i> null	Overexpressed	2	16	0.5

tobramycin exposure, as measured by C_{\max} and AUC_{0-24} .

Residual bacterial concentrations in lungs for treated vs. control rabbits were 6.26 ± 0.96 \log_{10} CFU/g ($n = 18$) vs. 8.13 ± 0.90 \log_{10} CFU/g ($n = 13$), 6.00 ± 1.05 \log_{10} CFU/g ($n = 10$) vs. 8.38 ± 1.06 \log_{10} CFU/g ($n = 6$), and 7.25 ± 1.24 \log_{10} CFU/g ($n = 9$) vs. 8.79 ± 0.62 \log_{10} CFU/g ($n = 4$), for rabbits infected with strains PAO1, 11B and MutGR1, respectively (Fig. 1). For each of the three groups of treated animals, the standard human-like tobramycin regimen was effective for pulmonary infection when compared with controls without treatment ($p < 0.05$ for each pair). The bacterial burdens in the animals receiving antibiotic treatment were similar to those observed in humans and (e.g.) in cystic fibrosis patients treated with intravenous antibiotics [18].

Spleen infection reflects bacteraemic events, and there was no difference, with respect to the mean bacterial concentration in spleens, between

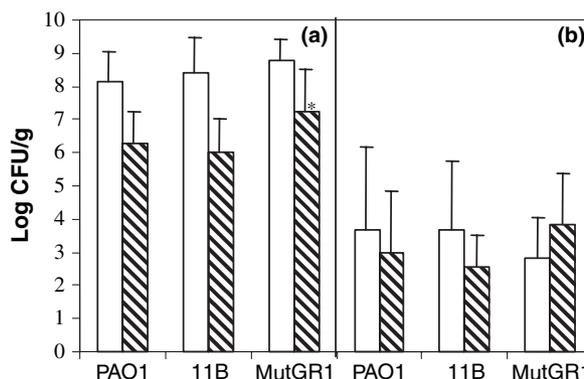


Fig. 1. Residual bacterial concentrations (\log_{10} CFU/g) in (a) lungs and (b) spleen of rabbits infected with *Pseudomonas aeruginosa* strains exhibiting varying expression levels of the MexXY efflux system (see Table 1). Treatment was for 48 h in a model of a human regimen of intravenous tobramycin (dashed bars, equivalent to 10 mg/kg once-daily in humans) in comparison with untreated controls (white bars). *Strain MutGR1 $<$ PAO1 and 11B, and all treated vs. untreated animals, $p < 0.05$.

treated and untreated rabbits in any group, as defined by the infecting strain. Tobramycin treatment did not reduce the spleen infection rate (72.1% at 48 h).

Overall, 48-h survival was significantly greater for animals receiving tobramycin treatment (100% for treated rabbits vs. 81.1% for untreated rabbits; $p < 0.01$), with no significant difference between groups as defined by the infecting strain. Bacterial concentrations in the lungs and spleen were associated significantly with mortality in univariate analysis (risk ratio 1.57, p 0.008, and risk ratio 9, $p < 0.01$, respectively); however, in multivariate analysis, pulmonary bacterial concentration was the only parameter associated significantly with mortality (risk ratio 5.8, $p < 0.01$). This is consistent with previous clinical studies highlighting the contrast between poor antimicrobial efficacy and improved survival [1,19–21].

In-vivo impact of the MexXY efflux system

Colonies were recovered on selective media from four control rabbits and four treated rabbits infected with wild-type strain PAO1. All of the colonies obtained from control rabbits had a wild-type antibiogram, whereas colonies with the MexXY phenotype, as defined by antibiogram, were retrieved from three treated rabbits (P35, P55 and P74) and had a gentamicin MIC of 16 mg/L. The pulmonary concentrations for these animals were 4.48, 1.39 and 1.40 \log_{10} CFU/g, with total residual bacterial concentrations of 7.59, 7.38 and 6.35 \log_{10} CFU/g lung, respectively (Fig. 2). The frequency at which resistance appeared in *Pseudomonas* in treated animals (3/14, 21%) is similar to the figure of 26% reported for treated humans [22]. After subcultures on antibiotic-free media, this MexXY phenotype appeared to be transient for all nine colonies recovered from the first rabbit (P35), but was stable for the colonies recovered from the two other treated rabbits. This suggests an adaptive resistance phenomenon in rabbit P35, with a MexXY overexpression rate of $c. 10^{-3}$, whereas stable mutational events occurring at a lower frequency of $c. 10^{-5}$ – 10^{-6} seem to have been selected in the colonies from rabbits P55 and P74 [5].

The impact of MexXY overexpression on the efficacy of tobramycin was assessed by compar-

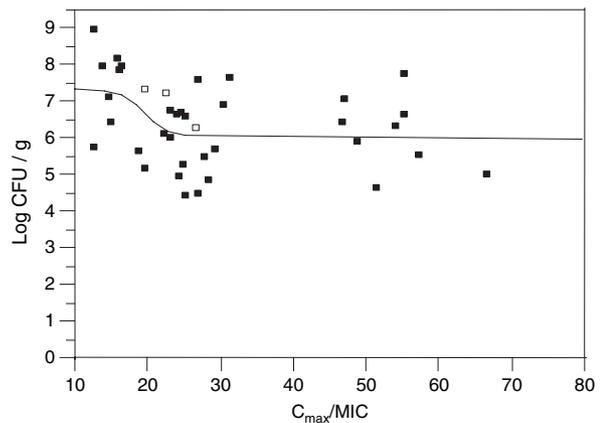


Fig. 2. Relationship between C_{\max}/MIC ratio and the residual bacterial concentration (\log CFU/g) obtained with *Pseudomonas*-induced pneumonia in rabbits treated with a 2-day human-like regimen of intravenous tobramycin. Open squares represent animals in which overexpression of the MexXY system was detected in bacteria from the lungs (see text). The black curve represents the regression line (E_{\max} model, p 0.05).

ing the residual bacterial concentrations in treated rabbits. Surprisingly, there was no significant difference in terms of residual bacterial concentrations between treated rabbits infected with the *mexX*-deleted strain 11B and treated rabbits infected with the wild-type strain PAO1. This suggests that MexXY efflux is of low significance and does not account for the low antimicrobial efficacy of tobramycin in rabbits infected with strain PAO1 in the present model. This contrasts with previous in-vitro and ex-vivo studies that have hypothesised that the MexXY efflux system could be a major mechanism involved in in-vivo resistance to aminoglycosides [2,3,5,23]. However, residual pulmonary bacterial concentrations in the present study were significantly higher in treated rabbits infected with strain MutGR1 than in both other groups, suggesting that overexpression of MexXY is responsible for the decreased antimicrobial efficacy of tobramycin. Thus, overexpression of this efflux system may be of concern in a clinical setting with longer aminoglycoside treatment periods where there is a higher risk of occurrence of resistance and therapeutic failure [24].

Pharmacodynamic analysis

Exposure to tobramycin was the same for the three groups (AUC_{0-24} 120 ± 24 , 115 ± 15 and

106 ± 26 (mg h)/L for animals infected with strains 11B, MutGR1 and PAO1, respectively). Following pharmacodynamic analysis, two pharmacodynamic–pharmacokinetic indices were poorly, but significantly, associated with tobramycin efficacy, as measured by the residual load of *Pseudomonas* in lungs, namely C_{\max}/MIC (r^2 0.25, $p < 0.05$; Fig. 2) and $\text{AUC}_{0-24}/\text{MIC}$ (r^2 0.18, $p < 0.05$), thereby confirming the concentration-dependent effect of tobramycin. It is of note that overexpression of MexXY occurred within the maximal selection window, i.e., between the C_{\max}/MIC values of 19 and 27. At higher values there was a plateau with no further decrease in bacterial load, which is in agreement with data published previously [25,26].

Conclusions

In contrast to previous studies performed in the planktonic/aerobic phase, in which the tested strains were all tobramycin-susceptible *in vitro*, the model system described in the present study indicates that the MexXY efflux system is of low significance and is not responsible for the poor antimicrobial activity of tobramycin in rabbits infected with the wild-type PAO1 strain. However, MexXY overexpression contributes to the increase in resistance to tobramycin observed in rabbits infected with strain MutGR1. Thus, other mechanisms of resistance to aminoglycosides must be involved, and these may have a greater impact *in vivo* than the MexXY efflux system in *P. aeruginosa* infections. Furthermore, although a concentration–effect relationship was confirmed in the present study, tobramycin had a relatively low level of efficacy in reducing the bacterial load in lungs, although there was a significant improvement in survival, probably associated with limited bacterial dissemination during treatment, as suggested by the splenic bacterial loads. The pulmonary bacterial reduction was *c.* 2 log₁₀ CFU/g, roughly inversely proportional to the MIC, which is consistent with results obtained in other animal models of *P. aeruginosa* pneumonia, although these models did not use regimens similar to those used in humans [27–30]. The bacterial reduction in lung tissue is also consistent with observations from clinical studies [20,31,32].

Many factors could explain the difficulty in eradicating *P. aeruginosa* strains causing pneumonia. Local hypoxia [33–35] and low pH [36]

may partly account for weak antimicrobial activity, since histopathological examination confirmed the presence of ischaemic lesions and multiple micro-abscesses. These lesions are similar to those seen in humans [37–39]. Tissue concentrations of tobramycin have not been determined because of technical difficulties, but tissue structure alterations may result in lower tissue concentrations so that the MIC for the infecting organism is not reached. In addition, observations of lung sections revealed that *P. aeruginosa* formed bronchial plugs and clusters/microcolonies in both treated and untreated animals, and the formation of such biofilms may explain *in-vivo* resistance to antibiotics [34,40,41]. The relatively poor efficacy of human tobramycin regimens may be explained by increased bacterial impermeability [42], by modification of periplasmic glucans [43], and by metabolic limitation and adaptation [33,44–48], with all of these factors contributing to increased MICs in biofilms [49–53], while the different efflux-pump systems, including MexXY, contribute only partially to classic antibiotic resistance [50]. Thus, the present results obtained with the model are consistent with existing knowledge [35,40,52,54–56], and the model seems to be well-suited for investigations of other anti-pseudomonal therapeutic strategies.

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