Increased Susceptibility of *Pseudomonas aeruginosa* to Macrolides in Biologically-relevant Media by Modulation of Outer Membrane Permeability and of Efflux Pump Expression

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Abstract (revised)

Background: *P. aeruginosa* (PA) is reported as intrinsically resistant to macrolides when tested by microdilution in broth according to CLSI guidelines. We previously showed (ICAAC 2010 poster D-751) that MICs are drastically decreased when measured in media for eucaryotic cell culture [RPMI], serum, or BAL. We have now studied the underlying mechanisms.

Methods: All experiments were performed with ATCC PAO1 strain exposed to azithromycin (AZM) or clarithromycin (CLR) and compared CA-MHB to RPMI, human serum, or mouse BAL. MIC were determined by microdilution. Kill-curves were performed over 24 h. Outer membrane (OM) permeation was assessed by evaluating the activity of combinations of AZM with agents altering outer membrane permeability using the checkerboard method. Macrolide accumulation was evaluated using ^{14}C-CLR (no radiolabelled AZM available). Expression of MexAB-OprM and MexXY-OprM was measured at the mRNA level by RT-PCR.

Results: MIC (mg/L) of AZM were 256 in CA-MHB, 16 in BAL, 8 in a 1:1 mixture of CA-MHB:serum, and 2 in RPMI. MIC of CLR were 512 in CA-MHB and 16 in RPMI. AZM was static in CA-MHB but bactericidal in RPMI (12 h incubation at 16 mg/L). Outer membrane permeabilizing agents were synergistic with AZM into MHB but not RPMI. Accumulation of CLR (1 mg/L) in PAO1 was 1.2 (4 h) and 1.7 (8 h) ng/mg of protein in CA-MHB but 2.3 (4 h) and 4.1 (8 h) ng/mg of protein in RPMI. *mexA* and *oprM* expression were markedly decreased (2 x and 4 x, respectively) in PAO1 maintained 8 h in the presence of 1 mg/L AZM in RPMI vs. CA-MHB.

Conclusions: The increased activity and bactericidal effect of macrolides towards PA in eucaryotic cell culture media and fluids from mammalian organisms could be due to an alteration of the OM integrity. This could favor the entry of the antibiotic within the bacteria, where it may repress the expression of efflux systems (contributing to a further increase of its own accumulation) resulting in a larger saturation of the ribosomal target. Because RPMI medium more closely mimics the composition of body fluids than CA-MHB and because similar effects were observed in serum or BAL, our data may have clinical significance.

References

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Background and aim

Pseudomonas aeruginosa, a major pathogen in pulmonary infections of cystic fibrosis or ICU patients, is considered as resistant to macrolides (2). However, macrolides, and AZM in particular, are widely used in such patients and contribute to reduce symptoms associated with infection and/or inflammation (3, 4). The mechanisms responsible for this activity remain unclear and may include anti-inflammatory effect (5), inhibition of the production of virulence factors by *P. aeruginosa* (6), and/or a direct effect on the outer membrane of the bacteria (7).

In this context, we have previously showed that the activity of macrolides against *Pseudomonas* is highly dependent of the medium (broth versus media used for eukaryotic cell culture, which better mimics biological media).

We have now studied the underlying mechanisms by examining the activity and accumulation of macrolides, the outer membrane permeability and expression of efflux systems into MHB and RPMI.

Methods

Bacterial strain and susceptibility testing. All experiments were performed with *P. aeruginosa* strain ATCC PAO1. MICs were measured by microdilution in MH broth or in RPMI medium (used for eucaryotic cells culture) supplemented with 10% of fetal calf serum, in MH broth supplemented by increasing amounts of serum, or in bronchoalveolar lavage fluid from non infected mice (BAL).

Kill-curves. Bacteria (10^8 CFU/mL) were cultivated in MHB or RPMI in presence of AZM (0, 1, 16, 128 mg/L) for a 24 h period. Every 4 h, bacterial suspension was diluted if necessary and plated on agar for counting after 24 h of incubation. To examine the role of media on susceptibility, suspensions were also harvested after 4 h of incubation and resuspended in other fresh medium.

Checkerboard. The activity of AZM combined with outer membrane disrupting agents was evaluated with checkerboard method. The first antibiotic of the combination was serially diluted along the ordinate (in MHB or RPMI), while the second drug was diluted along the abscissa in the seame medium. ΣFICs were calculated as follows: Σ FIC = FIC A + FIC B, where FIC A is the MIC of drug A in the combination/MIC of drug A alone, and FIC B is the MIC of drug B in the combination/MIC of drug B alone. The combination is considered synergistic if Σ FIC is ≤0.5, indifferent if 0. 5< Σ FIC < 2,and antagonistic if Σ FIC is ≥2.

CLR accumulation. Macrolide accumulation was evaluated using ^{14}C-CLR (no radiolabeled AZM available) in MHB and RPMI for 4 and 8 h

Expression of efflux systems. Expression of *mexA, mexX,* and *oprM* was measured at the mRNA level by RT-PCR in samples collected from bacteria incubated for 4 or 8 h in MHB or RPMI in the presence of AZM.

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Results

MICs of AZM against P.a. in different media

Antibiotics	MHB	RPMI	BAL	MHB/FCS	MHB/HS	MHB/RPMI
AZM	256	2	16	8	8	4
CLR	512	16	ND	64	ND	128

Table 1: MICs of AZM and CLR for PAO1 in different media: MHB, MHB cation adjusted; RPMI supplemented with 10 % fetal calf serum, BAL : broncho-alveolar fluid from non infected mice, MHB/FCS: 1:1 mixture of CA-MHB: fetal calf serum, MHB/HS: 1:1 mixture of CA-MHB: human serum MHB/RPMI: 1:1 mixture of CA-MHB: RPMI, ND: non determined.

Kill-curve of AZM against P.a. in different media

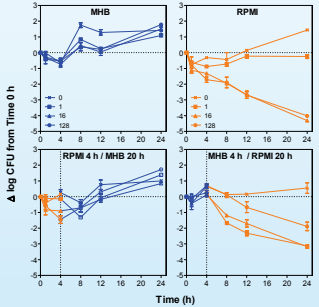


Figure 2: Kill-curves of AZM on PAO1 strain in MHB and RPMI. Bacteria are incubated with the indicated concentrations of azithromycin in MHB or RPMI (top panel); or in one of this medium for 4 h, centrifuged and resuspended in the other medium for 20 h (bottom panels)

✓ AZM is bactericidal in RPMI medium
✓ This effect is reversible and develops in 4-8 h

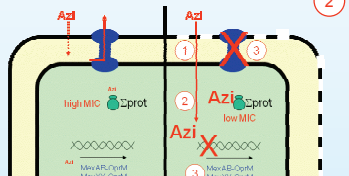
1 Outer membrane permeability in different media

combined compound	MHB MIC	MHB ΣFICs AZM+X	MHB comb.. effect	RPMI MIC	RPMI ΣFICs AZM+X	RPMI comb. effect
AZM	256 mg/L	-	-	2 mg/L	-	-
CST	2 mg/L	0.52 ± 0.03	S/I	2 mg/L	0.67 ± 0.14	I
CSA-13	4 mg/L	0.23 ± 0.04	S	4 mg/L	0.79 ± 0.19	I
EDTA	20 mM	0.07 ± 0.01	S	5 mM	1.75 ± 0.35	I

Table 2: Effect of combining azithromycin with outer membrane-disrupting agents on MIC in MHB or in RPMI. FIC < 0.5: synergy (S) and FIC > 0.5: indifference (I)

✓ Agents disrupting the outer membrane are synergistic with AZM in MHB but indifferent in RPMI

2 Accumulation of CLR in different media



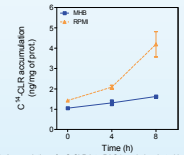


Figure 4: Accumulation of ^{14}C-CLR into PAO1 strain incubated in MHB or RPMI.

✓ clarithromycin accumulates to higher level when PAO1 is cultivated in RPMI

3 Expression of *mexA*, *mexX* and *oprM*

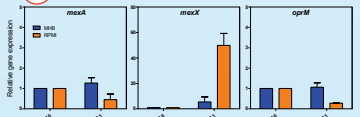


Figure 5: Expression of *mexA, mexX, oprM* in MHB or RPMI in presence or not of 1 mg/L AZM.

✓ *oprM* expression is downregulated by azithromycin in RPMI

Conclusions

The susceptibility of *P.aeruginosa* to macrolides is highly dependent on the culture medium. Our data suggest that some sort of permeabilization of thje OM takes place in RPMI, which allows for an increased accumulation of macrolides into bacteria. Once inside the bacteria, macrolides may exert their antibiotic activity on ribosome and downregulate the expression of the outer membrane protein OprM, which further contributes to increase their accumulation. Because RPMI medium and serum more closely mimic the composition of body fluids than CA-MHB, our data may have direct clinical significance.

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