Activity of tobramycin in combination with clarithromycin against *Pseudomonas aeruginosa* biofilms in an artificial sputum medium (ASM) model.

Eugénie Bassères,[1] Julien M. Buyck,[1] Hamidou Traore,[2] Paul M. Tulkens,[1] Francis Vanderbist,[2] and Françoise Van Bambeke.[1]

[1] Pharmacologie Cellulaire et Moléculaire, Louvain Drug Research Institute, Université catholique de Louvain, [2] SMB Laboratories; Brussels, Belgium.

Contact: francoise.vanbambeke@uclouvain.be

218

Introduction

Biofilms are considered as a major reason for persistence of lung infection in cystic fibrosis patients. In this environment, *P. aeruginosa* are mucoid (a phenotype in which an extracellular matrix of alginate is being produced), sessile, and metabolically less active. This lifestyle confers a higher resistance to antibiotics compared to planktonic bacteria [1]. Among anti-pseudomonal antibiotics, the aminoglycoside tobramycin is one of those available for pulmonary administration, and therefore widely used for controlling infective exacerbations in cystic fibrosis patients.

Macrolides are considered as inactive against *P. aeruginosa*, showing high MICs in standard medium (Mueller Hinton Broth, MHB). However, these antibiotics are often prescribed to cystic fibrosis patients, and improve their respiratory function. This positive effect is ascribed to non-antibiotic effects on the host (anti-inflammatory activity [2]) or on the bacteria (inhibition of quorum sensing [3]). Yet, it has been previously shown in our laboratory that macrolides regain activity on *P. aeruginosa* in media mimicking the infection site like bronchoalveolar lavage fluid [4].

In order to further investigate macrolide activity in cystic fibrosis, we developed a biofilm model with bacteria growing in an artificial medium mimicking the physico-chemical properties of the lung mucus during the disease (ASM; artificial sputum medium [5]). Indeed, the viscosity and components of this medium increase the ability of bacteria to attach to each other and to the surrounding surface, leading to biofilm formation.

Aim

To examine the effect of tobramycin and clarithromycin (as an exemplative macrolide) alone or in combination on
- biofilm formation
- eradication of preformed biofilms

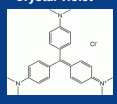
using in parallel
- the standard medium (MHB) and ASM
- a normal phenotype and a mucoid strain of *P. aeruginosa*

Methods

Strains: ATCC PAO1 and ATCC 39324 (stable mucoid).

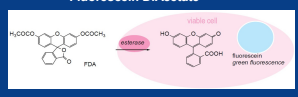
Biofilms were grown in 96 well-plates in ASM or Mueller Hinton Broth (MHB).
- ASM (1L): mucin 10g, DNA 4g, diethylene triamine pentaacetic acid 5.9mg, casaminoacids 5g, NaCl 4g, KCL 2.2g, tris base 1.81g, 5ml egg yolk emulsion.
- Antibiotic effect on biofilm formation was examined after 48 h incubation (drugs added at the time of inoculation).
- Antibiotic effect on mature biofilms was tested on 12-day old biofilms exposed during 48h to antibiotics (0.05 to 50 µg/mL tobramycin or/and 1 to 50 µg/mL clarithromycin).
- Biofilm biomass was evaluated by crystal violet staining and bacterial viability by fluorescein diacetate assay (6).

Crystal violet



Crystal violet binds to negatively charged surface molecules and to polysaccharides in the extracellular matrix. It stains living and dead bacteria plus the matrix allowing the quantification of the **biofilm biomass.**

Fluorescein DiAcetate



The quantification of **biofilm viability** was based on bacteria ability to convert non-fluorescent fluorescein diacetate into highly fluorescent fluorescein by non-specific esterases.

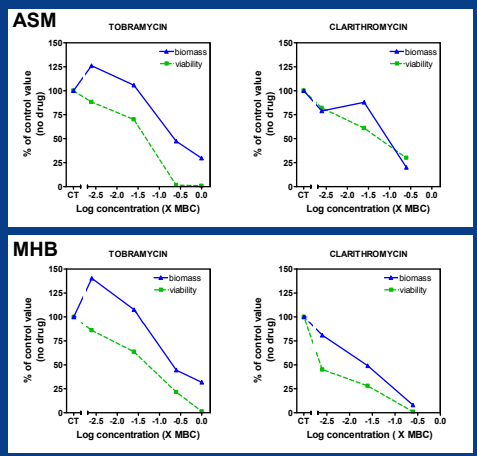
Results

Table 1: Minimal Bactericidal Concentrations (MBCs) of antibiotics as measured in ASM or in CA-MHB

medium	MBC (mg/L)			
	Tobramycin		Clarithromycin	
	PAO1	39324	PAO1	39324
ASM	5	50	≥1024	≥1024
MHB	0.5	0.5	≥1024	≥1024

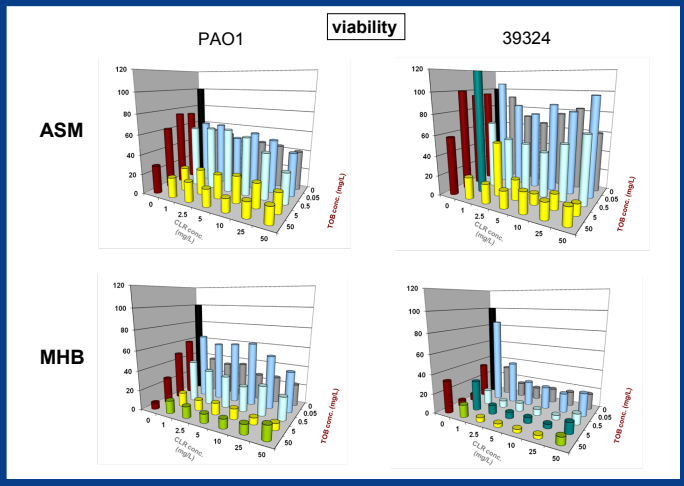
Tobramycin activity was decreased in ASM; clarithromyin was inactive in both media.

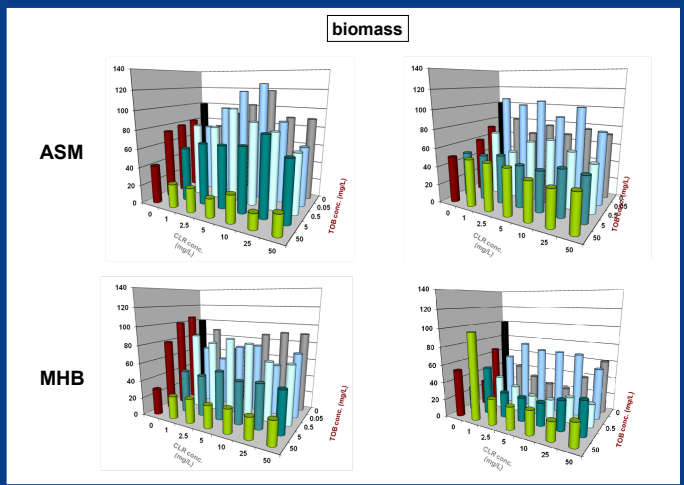
Figure 1: Effect of tobramycin and clarithromycin on biofilm formation by PAO1 after 48 h of incubation



Biofilm formation was prevented by both antibiotics at subMBC concentrations in both media.

Figure 2: Activity of tobramycin and clarithromycin in combination against 12-day old P. aeruginosa biofilms (wild-type and mucoid phenotypes) after 48 h incubation.
Bars in yellow correspond to combinations of concentrations where a significant synergistic effect is observed.





- Antibiotics alone were more active in MHB than in ASM and against viability than against biomass.
- Towards viability, a clear synergistic effect was observed when clarithromycin (any concentration) was combined with tobramycin at 5 or 50 µg/mL in ASM; this effect was less clear in CA-MHB where tobramycin was already highly effective alone.
- Towards biomass, combining clarithromycin to tobramycin did not bring significant improvement in activity.

Conclusion

- Although poorly active on planktonic bacteria, clarithromycin is as potent as tobramycin to impair biofilm formation, presumably due to its inhibitory effect on quorum sensing.
- Clarithromycin increases tobramycin activity against viability of *P. aeruginosa* (especially for a mucoid strain) growing in biofilms in a medium representative of cystic fibrosis patients' sputum.
- These data further document that eukaryotic media can reveal specific anti-pseudomonal effects of macrolides; they also rationalize the interest of macrolide administration in cystic fibrosis patients.

References

(1) Ciofu et al, FEMS Immunol Med Microbiol. 2012;65:215-25
(2) Labro; J Antimicrob Chemother 1998; 41(Suppl B):37–46
(3) Yousef and Jaffe, Paediatr Respir Rev 2010; 11:108–14
(4) Buyck et al, Clin Infect Dis 2012; 55:534-42
(5) Sriramulu et al, J Med Microbiol 2005; 54(Pt7):667-76
(6) Wanandy et al, J Microbiol Methods 2005;60:21-30