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**Evaluation of long term co-administration of tobramycin
and clarithromycin in a mature biofilm model of cystic
fibrosis clinical isolates of *Pseudomonas aeruginosa***

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Abstract

Pseudomonas aeruginosa colonization and chronic lung infection, associated with biofilm formation, is the major cause of morbidity and mortality in CF patients. Therefore there is an urgent need to develop alternative ways to treat biofilm associated clinical infections.

We performed a kinetic study over 28 days of a twice daily co-administration of the antibiotics tobramycin and clarithromycin on 12-day old, mature *P. aeruginosa* biofilms - formed on microplate pegs - for 23 clinical isolates of various phenotypes and genotypes to simulate the treatment of CF patients with inhaled tobramycin through aerosolisation (Tobi®). Drug activity was assessed by enumeration of persistent bacteria before, during and after treatment. We chose a mature (12-day old) biofilm model because a previous study suggested that such a biofilm was a more realistic *in vitro* model than 24-hour old biofilm.

A synergistic activity of the drug combination was confirmed on biofilms of 9/23 isolates of *P. aeruginosa*. Of these 9 isolates, a total destruction of the biofilm was observed for 5 of them. The combination treatment was superior or equivalent to the treatment with tobramycin alone as an activity was observed on 47.8% of the isolates with the combination versus 26.1% with tobramycin alone. There was no correlation observed between drug susceptibility profiles and the phenotype or the genotype of the isolates.

Keywords: *Pseudomonas aeruginosa*, mature biofilm, synergistic activity, treatment simulation, cystic fibrosis

1. Introduction

The major cause of morbidity and mortality in CF is chronic lung disease caused by a vicious cycle of infection and inflammation that leads to progressive deterioration in pulmonary function, respiratory failure and death¹. In particular, patients suffer from infection due to opportunistic *Pseudomonas aeruginosa*. *P. aeruginosa* overproduces an alginate exopolysaccharide within the CF lung, which appears to form a protective barrier around the bacterial cells and limits exposure to oxidative radicals, antibiotics and phagocytes². Bacterial biofilms play a relevant role in persistent infections which are rarely eradicated with antimicrobial therapy.

In a previous work we showed that an *in vitro* model of mature biofilm was more appropriate to mimic *in vivo* conditions in CF patients and we demonstrated a synergistic activity of a tobramycin/clarithromycin combination on biofilms of *P. aeruginosa* PAO1 and a clinical strain PYO2 using a kinetic study of a twice-daily co-administration over 9 days of antimicrobial agents on 12-day old biofilms³. In the present study, we tested 23 clinical isolates of various phenotypes and genotypes in order to confirm the synergistic activity of the combination tobramycin/clarithromycin on a broader sample of isolates. The possible correlation between susceptibility of the biofilms to the treatment and the phenotype and genotype of the isolates was also investigated.

2. Materials and Methods

2.1. Antimicrobial agents

We used micronized tobramycin (Teva, Petach Tikva, Israel) and clarithromycin (Teva). Stock solutions (10 mg/mL) of tobramycin were prepared in sterile water. For the preparation of clarithromycin stock solutions (2 mg/mL), the product was dissolved in methanol and completed with pH 6.5 phosphate buffer. Stock solutions were stored at -20°C until use. The tested concentrations of antibiotics were 4 µg/ml for tobramycin and 200 µg/ml for clarithromycin.

2.2. Bacterial strains

P. aeruginosa clinical isolates from sputum of 23 CF patients from diverse origins (Belgium, Germany, UK, the Netherlands) were tested. The isolates and epidemic strains⁴⁻⁶ included six mucoid phenotypes (MC075-450457, MC086, MC093, MC099-450467, MC110 and MC278) and seventeen non-mucoid phenotypes (Clone C, LUH 7752, MC039, MC075-350649, MC075-450456, MC084, MC096, MC099-450524, MC116, MC142, MC161, MC178, MC305, MC325, PHLS 8916, PHLS 8959 and PHLS 8960) and their genotypes were established using AFLP⁷. Identification of the strains was confirmed by tDNA-PCR⁷ and the API 20 NE system (bioMérieux, Marcy-l'Etoile, France).

Bacterial suspensions were made from fresh sub-cultures and aliquots were stored at -20 °C in glycerol until use. Before use, bacterial suspensions were spread onto Mueller-Hinton solid medium and incubated at 35 °C for 24 hours.

2.3. Genotyping

Genotyping was carried out by means of selective restriction fragment amplification (AFLP) as described previously⁷. Using high resolution capillary electrophoresis the fingerprints were immediately digitized and cluster analysis was performed.

2.4. Inoculum preparation for biofilm study

The inoculum was prepared in CAMHB at a concentration of $\sim 10^8$ colony-forming units (CFUs/mL) as described in a recent study³.

2.5. Biofilm formation

Biofilms were formed in 96-well microplates (Nunc Micro Wells Plates[®]) as described previously³. The plates were covered with lids presenting 96 pegs on which the biofilm can build up. The medium was changed daily from day 1 to day 12 in order to promote biofilm formation and maturation.

2.5. Kinetic study of antimicrobial agents co-administration to 12-day old biofilms

The kinetic study was performed as described previously³. The effect of antimicrobial drugs on preformed biofilm was studied on all 23 clinical isolates. After formation of mature biofilms, the pegs were removed, rinsed and put in contact twice daily during 28 days with tobramycin at 4 $\mu\text{g/mL}$ and clarithromycin at 200 $\mu\text{g/mL}$ alone or in combination in a new microplate. Enumeration of bacteria was also realised as described previously³. The logarithmic decrease of CFUs from antibiotic-treated biofilms was calculated using the following formula: $\text{logarithmic decrease} = [\log(\text{CFUs}_{\text{positive control}}) - \log(\text{CFUs}_x)]$; whereby x corresponds to the tested antimicrobial agent alone or in combination.

All tests were done in triplicate. All graphical evaluations were made using GraphPad Prism 4.03 (GraphPad Software Inc. San Diego, CA, USA).

3. Results

3.1. Kinetic study of co-administration of antimicrobial agents to 12-day old biofilms

Following the repeated co-administration of the two antimicrobial agents to the 12-day old biofilms, three different behaviours were observed, as illustrated in Table 1.

Firstly, a synergy between tobramycin and clarithromycin was recorded for 9/23 biofilms (group S), with a logarithmic bacterial decrease ranging between 1.8 and 7.5.

Figure 1A shows the synergy between tobramycin and clarithromycin for isolate MC161. When tobramycin was used alone, we observed resistance during the whole treatment whereas with the combination tobramycin/clarithromycin we observed an important logarithmic decrease ($\log_{10} 5.3$) of the number of viable bacteria present in the biofilm.

Secondly, for 8/23 biofilms (group TW), the activity of the combination tobramycin/clarithromycin was similar to that of tobramycin alone (Figure 1B) and no significant logarithmic decrease of the number of cfu/mL was recorded (0-1.5).

Thirdly for 6/23 biofilms (group TS) tobramycin alone or in combination with clarithromycin eradicated all the biofilm, represented by a strong decrease of the number of cfu/mL ($\log_{10} 6 - \log_{10} 6.5$), such as for isolate MC099 (figure 1C). No bacteria could be cultured from the pegs after 28 days of treatment, suggesting that in this group the kinetic model applied had a bactericidal effect.

When considering the global response of the treatments the combination versus tobramycin alone showed a better antimicrobial activity (minimum 1 log reduction) for 10 strains and an identical activity for 13 other tested strains. Antimicrobial reduction higher than 4 logs during treatment was obtained for 11 strains with the combination compared to for 6 strains when tobramycin was administered alone. Overall the combination treatment was superior to the treatment with tobramycin alone as a marked activity was observed on 47.8% of the isolates with the combination treatment versus 26.1% with tobramycin alone (Table 1).

3.2. Drug susceptibility versus phenotype and genotype

Twenty-two of the 23 strains were genotyped using selective restriction fragment amplification as described previously⁷. The Dice algorithm was used to calculate a similarity matrix which was used to carry out cluster analysis by means of the Neighbour Joining algorithm. A graphic presentation of the relatedness among the strains according to AFLP-analysis is presented in Figure 4. Several highly similar fingerprints were observed, e.g. for the isolates Clone C, MC075-450456, MC075-450457 (mucoid) and MC093 (mucoid), but visual inspection of the fingerprints indicated several different peaks present, even between strains from the same patient, i.e. between the two MC075 isolates. These genotypically related isolates were both synergistically inhibited, unlike a third isolate of this patient (MC075-350649) with a completely different fingerprint, which was not inhibited by tobramycin alone or the combination. The two isolates from patient MC099, one mucoid and one non-mucoid, were indistinguishable from each other according to AFLP, but these strains showed a different behaviour with regard to their susceptibility to the antibiotics, i.e. MC099-450467 (mucoid) was strongly inhibited by tobramycin alone whereas MC099-450524 (non-mucoid) was synergistically inhibited.

4. Discussion

Tobramycin is one of the most effective antibiotics against *P. aeruginosa* infections in CF, however eradication of the infection remains barely achievable.

A treatment able to eradicate chronic *P. aeruginosa* infections would increase the survival of CF patients significantly⁸.

Therefore, improving the efficiency of tobramycin treatment of biofilm grown *P. aeruginosa* was one of the objectives of this study.

During previous studies^{3,9}, we developed a mature biofilm model formed on microplate pegs which seems more appropriate than planktonic and fresh biofilm models to simulate *in vivo* conditions as met in CF patients and we demonstrated that the combination of tobramycin/clarithromycin might offer a better treatment for CF patients. In this study, we developed a kinetic *in vitro* model of mature biofilm mimicking the treatment TOBI^{®10} (tobramycin inhalation solution) of patients, during which TOBI[®] is inhaled twice a day during 28 days with intervals as close as possible to 12 hours apart, and no less than 6 hours apart. We tested concentrations of antibiotics in line with reported drug deposition¹¹ in the lungs for tobramycin and clarithromycin.

Biofilms were considered as mature from 12 days onwards referring to their formation process. Indeed from the 9th day onwards the last stage of maturation is reached. It was previously established that after 12 days of formation, the biofilm reaches full maturation¹². Moreover, we decided to follow a 28 days regimen for the antibiotics to mimic the therapeutic reality better than with a single dose antibiotic on a 'young' biofilm. This approach was

inspired by the standard treatment with TOBI[®] (Novartis) tobramycin inhalation solution USP¹⁰.

We confirmed an improvement in tobramycin activity by co-administration with clarithromycin for some biofilms. An activity with a 4-logarithmic reduction was observed on 47.8% of the isolates with the combination versus 26.1 % with tobramycin alone. Establishing a correlation between the genetic profile of isolates and their behavior with regard to antibiotic treatment would allow to predict the efficiency of a treatment combining tobramycin and clarithromycin. However, AFLP-analysis showed that there was little correlation between the genotype of isolates and their behavior with regard to antibiotics, especially because two isolates from the same patient (MC099), both with an identical AFLP fingerprint, but one mucoid and one non-mucoid, behaved differently with regard to antibiotic treatment.

Furthermore, there was no correlation between mucoidy and antibiotic resistance, although mucoid strains seemed to be more susceptible to tobramycin or combination therapy than non-mucoid strains, i.e. only one of the 7 mucoid strains (MC093) responded weakly to both tobramycin and combination therapy, whereas 7 of the 16 non-mucoid strains were difficult to treat by both antibiotic regimens.

Interestingly, the three UK epidemic strains seemed to be susceptible to tobramycin alone (PHLS 8916 and PHLS 8959) or to the combination therapy (PHLS 8960), whereas the continental epidemic strains (German clone C and Dutch LUH 7752) were resistant to both tobramycin and the combination therapy.

The synergistic effect observed between tobramycin and clarithromycin could be the consequence of the ability of clarithromycin to increase the permeability of the biofilm¹³. Moreover clarithromycin seems to have multifactorial activity. Mitsuya *et al.*¹⁴ suggested that macrolides may have an inhibitory effect on the GDP-mannose dehydrogenase (GMD) production cycle, hence inhibiting the formation of biofilm. GMD is one of the constituents of bacterial biofilm that is known to be involved in the production of alginate. Other authors¹⁵ also hypothesize that clarithromycin could inhibit the synthesis of alginate.

To our knowledge, this kind of kinetic long-term treatment study based on mature biofilm has never been carried out before.

The results confirm previous findings³ that the coadministration of tobramycin/clarithromycin offers a considerable potential for the treatment of at least some biofilm forming *P. aeruginosa* strains in CF.

In the future, animal or human models will be needed to corroborate these data with a measurable clinical outcome and to establish whether this mature biofilm kinetic treatment model can be used to predict the clinical outcome of different therapeutic strategies

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Declarations

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Competing interests: The authors declare no conflict of interest for this study.

Ethical approval: The clinical isolates tested were part of a routine taking realized at Hospital Gent, Belgium, for the characterization of CF infection strains by different studies including this one and do not require ethic clearance.

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Legends

Table legends:

Table 1: Effect of the antimicrobial agents tobramycin and clarithromycin, alone or in combination, on 23 clinical isolates of *P. aeruginosa*

Figure legends:

Figure 1: Kinetic study of the effect of administration of the antimicrobial agents tobramycin and clarithromycin, alone or in combination, on 12-day-old biofilms of (A) *P. aeruginosa* MC161-550367, (B) *P. aeruginosa* MC093-450507 and (C) *P. aeruginosa* MC099-450467

Figure 2: Phylogenetic tree constructed on the basis of AFLP-fingerprints of the studied isolates

Figure 1: Kinetic study of the effect of administration of the antimicrobial agents tobramycin and clarithromycin, alone or in combination, on 12-day-old biofilms of (A) *P. aeruginosa* MC161, (B) *P. aeruginosa* MC093, and (C) *P. aeruginosa* MC099-450467

(A)

(B)

(C)

TOB: tobramycin

CLR: clarithromycin

CFU: colony-forming units

Table 1: Effect of antimicrobial agents alone and in combination on 23 clinical isolates of *P. aeruginosa*

Behaviour	Logarithmic decrease			Strain number	Phenotype	Patient's origin	Genotype cluster
	TOB	CLR	TOB+CLR				
S	+	+	++	MC075-450457 KL	Mucoid	SVZ-TH	(a)
S	-	-	+	MC086	Mucoid	RUG-JL	
S	-	-	+	MC110	Mucoid	ULB-COCL	
S	-	+	+	MC075-450456 BL	Non-mucoid	SVZ-TH	(a)
S	-	+	++	MC084	Non-mucoid	SVZ-JB	
S	-	-	+	MC099-450524	Non-mucoid	RUG-DS	b
S	+	+	+	MC116	Non-mucoid	ULB-DS	
S	-	+	++	MC161	Non-mucoid	KUL-KB	
S	-	+	++	PHLS 8960	Non-mucoid	Epidemic strain Manchester UK	
TS	++	+	++	MC099-450467	Mucoid	RUG-DS	b
TS	++	-	++	MC278	Mucoid	UCL-SF	NT
TS	++	-	++	MC096	Non-mucoid	KUL-LG	
TS	++	-	++	MC178	Non-mucoid	ULB-WAAL	
TS	++	-	++	PHLS 8916	Non-mucoid	Epidemic strain Midlands UK	
TS	++	+	++	PHLS 8959	Non-mucoid	Epidemic strain Liverpool UK	
TW	-	-	-	MC093	Mucoid	KUL-LM	(a)
TW	-	-	-	Clone C	Non-mucoid	Epidemic strain Germany	(a)
TW	-	-	+	MC039	Non-mucoid	RUG-BDW	
TW	-	-	-	MC075-350649 Mat	Non-mucoid	SVZ-TH	
TW	-	+	+	MC142	Non-mucoid	ULB-GS	
TW	-	-	+	MC305	Non-mucoid	RUG-AD	
TW	+	-	+	MC325	Non-mucoid	RUG-KDS	
TW	+	-	+	LUH 7752	Non-mucoid	Epidemic strain the Netherlands	

Legend:

- S synergy of tobramycin and clarithromycin
 TS tobramycin alone, strong decrease
 TW tobramycin alone, weak decrease
 (a) very similar but not identical fingerprints
 b strains belong to the same cluster, because of identical fingerprints
 TOB tobramycin
 CLR clarithromycin
 + Logarithmic decrease of the number of cfu/mL ≥ 1
 - Logarithmic decrease of the number of cfu/mL < 1
 ++ Logarithmic decrease of the number of cfu/mL > 4

Figure 2: Phylogenetic tree constructed on the basis of AFLP-fingerprints of the studied isolates