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The role of suboptimal concentrations of nebulized tobramycin in driving antimicrobial resistance in *Pseudomonas aeruginosa* isolates in cystic fibrosis – an *in vitro* study

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John E. Moore: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Validation; Visualization; Roles/Writing - original draft; Writing - review & editing

Beverley C. Millar: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Validation; Visualization; Roles/Writing - original draft; Writing - review & editing

Marika Ollman-Selinger: Methodology; Writing - review & editing

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This paper is for informational purposes only. The authors do not make any recommendations on treatments, devices or pharmacological preparations.

John E Moore is in receipt of an Investigator Initiated Study and consultancy fees from PARI Respiratory Equipment Inc., USA.

Beverley C Millar has received non-financial support from PARI Respiratory Equipment Inc during the course of the study.

Marika Ollman-Selinger is an employee of PARI GmbH.

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QUICK LOOK

Current Knowledge

Nebulized tobramycin is a first line therapy for the eradication and chronic suppression of the bacterial pathogen, *Pseudomonas aeruginosa* (PA) in people with cystic fibrosis (CF). Tobramycin is mainly delivered to the lungs by jet nebulizers which have varied performance characteristics. These differences in delivery efficiencies are based on aerosol characteristics such as respirable dose (RD) [mg], which is the amount of drug that can reach the lower airway, mass median aerodynamic diameter (MMAD) [μm], fine particle fraction (% <5μm), and fine particle mass [mg]. Poor nebulizer efficiency may lead to the delivery of sub-optimum concentrations of antibiotic to the lungs, which may adversely alter bacterial persistence and antibiotic susceptibility.

What This Paper Contributes To Our Knowledge

Sub inhibitory concentrations of tobramycin inhalation solution (TIS) emulating inefficient nebulizer delivery drives bacterial persistence and antibiotic resistance with PA *in vitro*. *In vitro* cycling of 28 days on and 28 days off of TIS improves PA susceptibility to tobramycin. Efficient nebulizer choice is important to minimize bacterial persistence and tobramycin antibiotic resistance development.
ABSTRACT

BACKGROUND: Antimicrobial resistance in *Pseudomonas aeruginosa* (PA) may be driven by exposure to suboptimal concentrations of tobramycin antibiotic delivered by less efficient nebulizers.

METHODS: PA isolates (N=114; 32 first+82 chronic) were challenged *in vitro* employing extrapolated peak and trough concentrations of tobramycin inhalation solution (TIS), corresponding to three nebulizers, PARI LC PLUS, Sidestream12NEB400 and MistyNeb2035G. Bacterial persistence and antibiotic susceptibility to tobramycin was determined following four TIS cycles:- (i) 28d ON, (ii) 28d ON+28d OFF (iii) 2x28d ON and (iv) 28d ON +28d ON+28d OFF.

RESULTS: All first isolates were eradicated at peak and trough concentrations except for the trough concentration corresponding to Sidestream 12NEB400 (bactericidal activity 87%). For chronic isolates, peak concentrations eradicated 88%, 90% & 92% and trough concentrations eradicated 43%, 62% & 85%, corresponding to Sidestream12NEB400, MistyNeb2035G and PARI LC PLUS, respectively. A statistically significant increase in antibiotic resistance (MIC) with sensitive, intermediate and resistant PA was noted following cycles (i)–(iv), at trough concentrations corresponding to Sidestream 12NEB400 and MistyNeb2035G. There was a significant reduction in tobramycin resistance following a 28d OFF cycle and no difference, following 1x28d ON versus 2x28d ON cycles.

CONCLUSION: This *in vitro* study showed that suboptimal concentrations of tobramycin drove increased antibiotic resistance, emulating standard cycles of ON/OFF inhaled therapy. This was evident at extrapolated tobramycin concentrations at trough levels corresponding to less efficient nebulizers, by (i). initially allowing for the survival of intermediate and resistant organisms, because nebuliser performance did not achieve critical MIC concentrations sufficient to eradicate the
organism, (ii). allowing the development of resistance in those cells that were able to survive the initial tobramycin challenge. Transferred to clinical practice, this means for people with CF on TIS treatment, it is important that clinicians employ an efficient nebulizer that helps mitigate an upward drift in antibiotic resistance, thereby protecting the clinical value of TIS within CF care.

**KEY WORDS:**

- *Pseudomonas aeruginosa*;
- tobramycin inhaled solution (TIS);
- Nebulizer performance;
- Antimicrobial Resistance (AMR);
- Bacterial persister;
- Cystic fibrosis
- Antibiotic cycling
INTRODUCTION

Cystic fibrosis (CF) is the most inherited autosomal recessive lethal genetic disease in the Caucasian population, accounting for approximately 70,000 people with CF globally and approximately 30,000 persons within the US. The basis for the disease is genetic mutations within the cystic fibrosis transmembrane conductance regulator (CFTR) protein, leading to absence or abnormalities of this protein, which results in problems in the cells’ ability to transport chloride ions across cell membranes. This results in sticky mucus which blocks the pancreatic duct, the gastrointestinal tract and alters airway clearance and antimicrobial defences within CF lungs.

Failure to clear mucus results in the entrapment of many environmental bacteria and fungi, particularly the bacterial pathogen, Pseudomonas aeruginosa (PA). This leads to a vicious cycle of infection and inflammation, ultimately resulting in increased morbidity and mortality. Therefore any treatments (oral, nebulized, iv antibiotics) and therapies (mucolytic and airway clearance techniques) that help eliminate PA from the CF airways will help reduce the microbiological burden of this organism and suppress pulmonary exacerbations, minimize respiratory inflammation and slow down any decline in lung function.

The Cystic Fibrosis Foundation (CFF) recommends inhaled tobramycin in mild, as well as in moderate-to-severe lung disease, based on evidence from three randomised controlled trials involving 234 patients, and one randomized cross-over trial, involving 1,110 patients that substantially benefited from treatment. Several other international cystic fibrosis Standards of Care Guidelines advocate tobramycin for maintenance therapy in chronic infection, as well as for eradication therapy on first isolation of PA. Inhaled tobramycin is available in two formulations, either as Tobramycin Inhalation Solution (TIS) or as a dry powder. Use of dry power tobramycin is limited to those who can properly demonstrate the technique and is also commonly used. Two Cochrane Reviews also support inhaled tobramycin as an antipseudomonal antibiotic, for both eradication and maintenance therapy.
To be clinically effective at killing PA, adequate concentrations of nebulized tobramycin must be delivered to the CF airways. More specifically, these concentrations need to be above the threshold antibiotic concentration, known as the minimum inhibitory concentration (MIC) of the new or chronically colonizing PA strain. Several factors can be responsible for the occurrence of sublethal (<MIC) tobramycin concentrations within the CF airway, as detailed in Figure 1. Unlike patient-related factors, such as adherence to nebulized tobramycin therapy or disease characteristics, device choice is an important aspect of CF therapy that clinicians can control. Clinicians can optimize delivery of TIS by selecting an efficient nebulizer based on published performance data.

Several studies have examined the effects of nebulizer efficiency on tobramycin delivery to the CF lungs. In a related Cochrane review investigating “Nebuliser systems for Drug Delivery in Cystic Fibrosis”, these authors concluded that there was variability in the performance of different nebulizer systems.

In the UK, nebulized tobramycin is approved specifically with the PARI LC PLUS nebulizer. In the US, the FDA has historically specified that tobramycin inhaled solution (TIS) should be administered via a PARI LC PLUS nebuliser, following the seminal paper by Ramsey et al. Presently, there are ten commercially available nebulized tobramycin formulations listed in the US FDA “Orange Book” (40th Edition), in addition to the United States Pharmacopeia. All of these compendia specify that TIS should be delivered by a PARI LC PLUS nebulizer. Whilst this is the case, healthcare providers and individuals with CF may opt to deliver nebulized tobramycin via an alternative nebulizer in part, because individuals may not always have access to the PARI LC PLUS. TIS is a common therapy in patients with chronic PA and is the third most common medication prescription after dornase alfa and hypertonic saline. In 2018, 70.2% of people with CF with PA (6 years of age or older) were taking inhaled tobramycin. In children, inhaled tobramycin was used therapeutically in 18.5% (<3 years, n=1,877) and in 22.1% of children aged 3 to 5 years (n=2,272). A recent US survey was conducted amongst CF patients and providers,
which indicated that from the 671 CF community responses from 48 states, PARI brand devices were the most commonly used (50%). The survey also showed that 46% of responders did not have enough nebulizer cups. Given these data and that 70.2% of those with PA used inhaled tobramycin, it is anticipated that there are individuals that are using nebulizers, other than the PARI LC PLUS to deliver TIS. A previous study compared the efficiency of 18 disposable nebuliser systems with two reusable nebuliser systems and demonstrated that none of the disposable nebulisers tested could be recommended as an alternative to the PARI LC PLUS nebuliser for tobramycin delivery due to lower lung deposition with the less efficient nebulisers tested (Supplementary Figure 1).

Antimicrobial resistance (AMR) is an emerging public health problem globally, however it is a particular problem in chronically infecting organisms of the airways in CF. In the US, rates of multidrug resistant (MDR) PA infection are greatest in older adolescents and adults with CF, probably reflecting cumulative exposure to antibiotics. Among those people with CF who had at least one bacterial culture in 2018, 7.5% were reported to have MDR-PA. Additionally, among people with CF with PA infection in 2018, 16.9% were reported to have MDR-PA. However, the CFF Registry reports that the clinical significance of such antibiotic resistance remains unclear. Elsewhere, increasing antibiotic resistance to tobramycin has been reported. In Northern Ireland, a recent study showed that tobramycin resistance increased significantly (p<0.0001) over the period 1996-2016.

The importance of inhaled antibiotics in CF has been well established, in terms of the clinical benefits derived from such therapies, with extensive information supporting its safety and efficacy. As inhaled tobramycin is considered a firstline antipseudomonal, it has been suggested that high local concentrations delivered by this route may help overcome resistance. It has previously been shown that exposure of suboptimal concentration of antibiotics may lead to
acquisition of antimicrobial resistance. We therefore hypothesized that suboptimal delivery of lower concentrations of tobramycin to the lungs by less efficient nebulizers may have an effect on the antibiotic susceptibility of PA.

Therefore, it was the aims of this study to examine:-

(i). the \textit{in vitro} survival of PA when challenged with extrapolated peak/trough concentrations of tobramycin emulating delivery by three nebulizer devices used in CF nebulized therapy,

(ii). the \textit{in vitro} drift of tobramycin susceptibility in PA when challenged with tobramycin emulating peak/trough nebulizer extrapolated concentrations with four treatment regimen combinations and

(iii). the \textit{in vitro} effect of 28 day OFF period on PA tobramycin susceptibility.
MATERIALS AND METHODS

A series of in vitro experiments were designed to emulate the delivery of nebulized tobramycin and a summary of the microbiological procedures employed in this study is shown in Figure 2.

Description of Pseudomonas aeruginosa isolates

This was entirely an in vitro study and did not involve the collection of specimens from any CF individual for the purposes of this study. All isolates employed in this study were obtained from an existing public bacterial isolate repository, the HSC Microbiology Isolate Culture Collection Repository (CF MicroARK) housed at the Northern Ireland Public Health Laboratory. No extra or additional requests for sputum were made from individuals with CF. As all PA isolates employed in this study were completely anonymous, no clinical patient identifiers or other information was available.

All experiments were performed at the Northern Ireland Public Health Laboratory, Belfast City Hospital, Belfast, UK. The identification of each isolate was confirmed by analysis using matrix-assisted laser desorption/ionization/time-of-flight mass spectrometry (MALDI-TOF). Prior to use, a purified single colony of each isolate was passaged three times, by subculturing on Columbia Blood agar (Oxoid CM0031, Oxoid Ltd., Basingstoke, UK), supplemented with 5% (v/v) defibrinated horse blood for 24h at 37°C, under aerobic conditions. In addition, the reference strain, P. aeruginosa WDCM000025 (ATCC™ 27853), was employed as a control.

Peak/Trough tobramycin concentrations used

As this was an in vitro study, nebulizers were not physically employed as such, peak and trough tobramycin concentrations are subsequently referred to those previously published (PARI LC PLUS) or extrapolated (Sidestream 12NEB400 and MistyNeb 2035G). The tobramycin
concentrations used in this study are shown in Table 1. In the case of the FDA-specified nebulizer for TIS delivery (PARI LC PLUS), the concentrations used were obtained from a pharmacokinetic safety study by Hubert et al.\textsuperscript{12}, where sputum and serum concentration deposition of TIS (300mg/5ml) delivered by the PARI LC PLUS nebulizer (PARI GmbH, Germany), [Pulmoaide compressor, USA] administered twice daily over 15 days was investigated in individuals with CF with chronic PA infection.\textsuperscript{12} In the case of two less efficient nebulizers, as reported by Vecellio et al.\textsuperscript{8}, (based on the fine particle mass being >20% lower), namely the Sidestream 12NEB400/wall air (Respironics, UK) and the Misty Neb2035G/Pulmoaid compressor (Cardinal Health, France), which are commonly used in the US, extrapolated tobramycin concentrations were determined as shown in Supplementary Figure 2.

**Description of tobramycin cycling regimens employed**

Four tobramycin cycling regimens (ARMS #1 - #4) were identified and adopted into the study protocol as detailed in Figure 3. An initial pilot study was undertaken with PA (N=20 isolates) to ascertain if PA was able to survive for 56 days (2 x 28 day cycles) incubation in nutrient broth. When switching from an “ON” cycle i.e. in the presence of tobramycin to an “OFF” cycle, an inoculum (10 μl) of incubated culture from the previous “ON” cycle with tobramycin, was subcultured into fresh nutrient broth (Oxoid CM1) (10ml), in the absence of tobramycin.

**Determination of initial tobramycin susceptibility**

All 114 PA clinical isolates, as well as the control \textit{P. aeruginosa} WDCM000025 (ATCC™ 27853), were initially screened for tobramycin susceptibility, using disk diffusion (tobramycin 10 μg disk) (Oxoid), in accordance with CLSI methodology and interpretive criteria,\textsuperscript{28} namely where zone size ≥ 15mm = sensitive (S); 13-14 mm = intermediate (I) and ≤ 12mm = resistant (R). Isolates were subsequently categorized into S, I or R groupings.\textsuperscript{28}
Determination of survival of PA isolates at Peak/Trough concentrations of tobramycin

The concentrations of tobramycin as detailed in Table 1 were prepared using Mueller-Hinton Broth No. 2 (MHB2) (Sigma, UK), in glass universals (7mls). To this, a fresh inoculum of each PA isolate was individually added to give an initial bacterial density, in accordance with Clinical and Laboratory Standards Institute (CLSI) methodology and the inoculated broth was incubated at 37°C for 24h. Following this, broths were examined visually to check for bacterial growth, as manifested by cloudiness/tubidity of the broth and at a further 24h. Positive broths were incubated as per the study arm conditions (Figure 3).

Determination of tobramycin susceptibility in Study ARMS #1 - #4

PA (N=38 isolates) consisting of eight sensitive (S) isolates (1st PA isolates) [CLSI MIC\textsubscript{TOB} \leq 4mg/L], 14 intermediate (I) isolates [CLSI MIC\textsubscript{TOB} = 8 mg/L] and 16 resistant (R) isolates [CLSI MIC\textsubscript{TOB} \geq 16mg/L] were employed. Isolates were co-cultured in MHB2 at each of the six tobramycin concentrations (from above), as well as in a control broth containing no tobramycin, in accordance with each of the study arms (#1 - #4). The MIC of each isolate at each tobramycin concentration in each study arm was determined in accordance with CLSI antimicrobial susceptibility test methodology, employing tobramycin E-test strips (MIC range 0.064 – 1024 mg/L) (Liofilchem SRL., Italy) and used in accordance with the manufacturer’s instructions.

Following tobramycin challenge in accordance with ARMS #1 - #4, MHB2 (10 μl) containing viable PA were subcultured in nutrient broth (10mls) (Oxoid CM1) and further incubated at 37°C, in accordance with the Study Arms (Figure 3).

Statistical analyses

The compliance of parameters to a normal distribution was assessed using the Shapiro-Wilk test and subsequently the EDISON-WMW: Exact Dynamic Programming Solution of the Wilcoxon-Mann-Whitney Test was applied to examine statistical significance.
RESULTS

Description of PA isolates and survival with extended culture

PA isolates (N=115) were employed in this study, consisting of 32 first isolates, 82 chronic isolates and the PA Reference strain, WDCM00025 (ATCC 27853). Results from a pilot study to investigate whether PA isolates could survive longterm incubation showed that they were able to survive in nutrient broth at 37°C for at least 56 days (Supplementary Figure 3).

This study showed that peak and trough tobramycin concentrations from the PARI LC PLUS and peak and trough concentrations with the Misty-Neb eradicated all first isolates (Figure 4a). However, while the extrapolated tobramycin peak concentration of the Sidestream nebulizer, eradicated all organisms, its extrapolated trough concentration was unable to eradicate a small proportion of these isolates (Figure 4a). When examining chronic isolates, none of the peak tobramycin concentrations from any of the nebulizers were able to eradicate all isolates (Figure 4b), although there was a positive correlation with tobramycin concentration (Supplementary Figure 6). Likewise, there was wide variation in the ability of the extrapolated tobramycin concentrations to eradicate chronic isolates at their respective trough concentrations, ranging from 85% bactericidal activity for the PARI LC PLUS to 43% bactericidal activity for the Sidestream (Figure 4b).

Tobramycin susceptibility in Study ARMS #1 - #4

PA isolates at the extreme ends of tobramycin susceptibility were exited from the study at this initial stage. Of the 114 original clinical isolates examined in this study, 62 were fully sensitive to the lowest concentration of tobramycin examined (7 mg/L) and were subsequently exited from the study. These isolates were exited at this stage as they would have been unable to survive the lowest concentration of tobramycin employed in subsequent study arms. A further seven highly resistant isolates had an initial tobramycin sensitivity of >1024 mg/L and were also exited from the study, as
the method of tobramycin susceptibility testing employed, namely quantitative E-test strips, had a maximum MIC value of 1024 mg/L, hence it would have been difficult to track changes in tobramycin susceptibility concentrations greater than the maximum value offered by the E-test strip.

Fifty two isolates were resistant to at least 7 mg/L tobramycin concentration, however 14 of these isolates were excluded from the continued study due to non-survival of the control sample or which became contaminated during the extended incubation durations of the study arms. A total of 38 PA isolates were subsequently followed through ARMs #1-#3.

Antibiotic susceptibility at each of the tobramycin co-culture concentrations within ARMs #1-3 are shown in Figures 5-7, respectively, where Figure 5 represents tobramycin susceptibility in S, I & R PA isolates (N=38), following 28 days ON tobramycin. Figure 6 represents tobramycin susceptibility in S, I & R PA isolates (N=38), following 28 days ON + 28 days OFF. Figure 7 represents tobramycin susceptibility in S, I & R PA isolates (N=38), following 2 x 28 days ON tobramycin.

In the case of isolates which remained viable following the incubation with subinhibitory concentrations of tobramycin, the antibiotic susceptibility to tobramycin decreased, resulting in statistically higher MIC values. This was consistent throughout all four arms of the study (Figures 5-7 & Supplementary Figure 4). Additionally, this was evident with all antibiotic susceptibility classification of isolates (S, I, R). The increase in MIC, although observed within all such classifications, was most notable in the sensitive and intermediate classes (Figures 5-7 & Supplementary Figure 4). Supplementary Figure 4 represents antibiotic susceptibility of total PA isolates following 2 x 28 days ON + 28 days OFF tobramycin.

The effect of incubation duration on tobramycin susceptibility in 40 PA isolates, in the absence of tobramycin showed a decrease in the tobramycin MIC, i.e. increased susceptibility (Supplementary
in vitro study, demonstrating that any increase in tobramycin resistance was a direct effect of tobramycin co-culture and not prolonged incubation. This effect may be attributed to a changing physiological environment of nutrient exhaustion, nutrient competition, accumulation of bacterial waste products and metabolic shutdown/pseudodormancy, where the cells are preparing to enter a dormancy stage in order to remain viable and metabolically inactive.

Supplementary Figure 6 represents antibiotic susceptibility to tobramycin following incubation of PA isolates (N=38) with tobramycin (7 mg/L), in particular, the effect of the 28 days OFF cycle on tobramycin susceptibility with 28 days ON + 28 days OFF versus 2 x 28 days ON cycles.

All statistical (p) values are detailed on the individual graphs and figures.
DISCUSSION

Less efficient nebulizers may be employed in the hospital setting for a cost saving measure or for convenience, particularly relating to nebulizer hygiene, which make other nebulizers appear more attractive.\(^3\) Additionally, many people with CF typically utilize cycled inhaled tobramycin as part of their routine treatment regimen in the home. If nebulizer supplies are not obtained from the CF clinic or are not specified in the prescription, different nebulizer types (i.e. single use disposables) may be obtained from home care suppliers or pharmacies. Additionally, inefficient aerosol compressors may be obtained that do not provide the same specifications as ones approved to deliver TIS. So even with the approved FDA nebulizer, there could still be efficiency issues if the compressor is inadequate.

Use of less efficient nebulizers may be adequate to deliver other nebulized CF medications, such as albuterol, however it can adversely impact when delivering concentration-dependent antibiotics such as tobramycin. Clinicians need to be aware of the delivery efficiency of the nebulizer being used to treat patients requiring tobramycin inhaled therapy, to ensure optimal concentrations of antibiotic reaches the lung. When these concentrations are not optimally achieved, reduced concentrations of antibiotic may have adverse consequences on bacterial persistence and antibiotic resistance, as demonstrated in this standardised controlled \textit{in vitro} study. Therefore, respiratory therapists should be aware of the significance of the \textit{in vitro} findings of the current study when considering choice of nebulizer for inhaled tobramycin therapy in individuals with CF.

Several factors can be responsible for exposure of PA to sublethal concentrations of tobramycin (Figure 1) including patient-related factors that are difficult to control, such as disease severity and individual adherence to nebulized treatments. The experimental approach of the current \textit{in vitro} study was designed to circumvent the complexities of the \textit{in vivo}, by simply examining \textit{in vitro}
susceptibility with one single variable, namely tobramycin concentration. Selection of a more efficient nebulizer to deliver higher concentrations of antibiotic to the CF lung, is one variable that can be controlled by clinicians to avoid sublethal concentrations that may encourage development of AMR. To date, there have been no reports, either *in vivo* or *in vitro* which has examined the potential correlation between low concentrations of tobramycin extrapolated from less efficient nebulizers and AMR. Therefore, the main aim of this *in vitro* study was to examine the effect of exposing clinical isolates of PA to sublethal concentrations of tobramycin inhalation solution (TIS), emulating poor nebulizer performance and to identify if such exposure resulted in altered bacterial persistence and increased antibiotic resistance to tobramycin.

**Effect on bacterial persistence**

It is important that nebulizers deliver sufficient concentrations of tobramycin that exceed the MIC of newly acquired PA isolates. Failure to deliver adequate nebulized concentrations of antibiotic to the CF airways will allow PA to persist and thereby evade eradication. This leads to chronic colonization and makes future attempts at eradication much less successful.

**Effect on antimicrobial resistance**

Antimicrobial resistance (AMR) is an important problem in managing individuals with CF, leading to reduced options of available antibiotic classes, which may lead to treatment dilemmas. AMR may manifest in *P. aeruginosa* at two stages, namely (i) on first isolation of the organism and (ii) during the chronic phase of infection. It is unusual that first isolates are resistant to antipseudomonal antibiotics, indicating that these isolates originate in the environment, where they are not exposed to such antibiotics and are sensitive to antipseudomonal antibiotics. However, occasionally first isolates may be resistant to certain antibiotics and this suggests acquisition of an organism from other people with CF or the healthcare environment and not as the consequence of a true environmental acquisition.
Once early colonisation has been established, there are several clinical scenarios which may allow AMR to develop, including (i). use of antibiotics in oral, nebulized/inhaled and IV formulations, (ii). duration and frequency of use of antibiotics and (iii). any clinical scenario which would potentially lead to exposure of the organism to sub-optimal concentrations of the antibiotic. These include poor patient compliance in taking antibiotics, poor antimicrobial stewardship practices and failure to deliver antibiotic at optimal concentration to the lungs due to the use of less efficient nebulizers (Figure 1).

When the MIC of PA organisms already exceeds the peak TIS concentration delivered by the nebulizer, then tobramycin no longer offers effective bactericidal activity, leading to persistence of PA and ineffective clearance/eradication. The clinical significance of such data emphasises the importance of nebulizer efficiency, to ensure that antibiotic concentrations delivered by the nebulizer are maximized, so that the concentrations delivered are greater than the MIC value of the infecting strain, thus optimising first isolate eradication and suppression of chronic PA isolates.

When MIC values increase due to the factors highlighted above, then inefficient nebulizers may no longer deliver antibiotic at sufficient concentrations to be bactericidal.

**Importance of cycling nebulized tobramycin**

On comparison of MIC values across ARMS #1 - #3, it was noted that the MIC after a 28 day ON/28 day OFF cycle of tobramycin was significantly lower than a single 28 days ON cycle (p=0.008) (Supplementary Figure 6). This is the first *in vitro* evidence that 28 days OFF cycle promotes an increase in tobramycin susceptibility, providing evidence in support of the conventional treatment regime of 28 days ON/ 28 days OFF. Historically, early tobramycin treatment regimens included a 28 days OFF cycle, with the concept of minimising the development of tobramycin resistance, but none of these early studies provided an evidence-base which helped prove the need for this OFF period to minimise resistance. This data substantiates the importance
of this OFF cycle for tobramycin. Antibiotic susceptibility was greatest at the end of the 28 days OFF cycle (Supplementary Figure 6), therefore, it is important to ensure that individual adherence to nebulized tobramycin is maximised when the next 28 days ON cycle commences. The aim is to exploit the vulnerability in MIC values created by the preceding 28 days OFF cycle.

Furthermore, many other treatment regimens employing antipseudomonal nebulised antibiotics other than tobramycin have copied the 28 days OFF period, without providing any evidence of what this means for resistance development with that particular antibiotic. Given that many of these other inhaled antibiotics, including colistin, aztreonam and levofloxacin belong to other classes of antibiotic other than the aminoglycosides, each with their own unique biology, including class-specific mode of action and associated mechanisms of antibiotic resistance, it is unwise to extrapolate the findings of this aminoglycoside to these other antibiotics, in terms of justification for their 28 days OFF cycle. Therefore, similar studies to the present study need to be performed on these other non-aminoglycoside antipseudomonal antibiotics, in order to substantiate claims for a 28 days OFF period and what this means for resistance development for these antibiotics.

Importance of nebulizer choice

Previous in vitro comparisons of nebulizers have demonstrated a wide variation in nebulizer delivery efficiency. An in vitro study of 20 jet nebulizer/air source combinations (18 disposable and 2 reusable) compared the delivery efficiency of TIS (300 mg/5 mL) and showed that the combination of the nebulizer, interface and air source can have a significant impact on the respirable dose of TIS to the lungs. It concluded that none of the disposable nebulizers tested could be recommended as an alternative to the PARI LC PLUS for tobramycin delivery, due to the lower respirable TIS doses with the other nebulizer/air source combinations. The Vecellio study did not investigate how these performance differences may have potential clinical consequences, such as bacterial persistence and antimicrobial resistance, which was the aim of our study.
Study limitations

Whilst the adoption of this in vitro study helps to circumvent several variables, as discussed above, it equally presents several limitations, as does any in vitro study, when attempting to interpret the data within the in vivo setting. As no in vitro or in vivo data exists, a controlled in vitro study offers the opportunity to singularly examine one variable, namely tobramycin concentration, which would be difficult to control in any clinical trial, due to multiple confounders. This current study therefore focused entirely on the in vitro effects on antibiotic susceptibility of PA isolates, when grown in the presence of sub-inhibitory concentrations of tobramycin. No nebulizers were employed to physically deliver tobramycin to the PA isolates, rather isolates were incubated in the presence of extrapolated tobramycin concentrations. Extrapolated concentrations were in turn derived from previously published fine particle mass experimental data, which had varying standard deviations. Furthermore, organisms were challenged at constant peak concentrations of tobramycin. We know that this is not the case in vivo, as previous PK/PD studies have shown the peak inhaled tobramycin concentration in sputum is achieved with the LC PLUS approximately 30 mins following first delivery and the concentration is reduced to approximately one third (approx. 750 v 250 μg/g sputum) 1.5 hours later. However notwithstanding these limitations, what this study does show is that sub-inhibitory concentrations of tobramycin have the potential to promote a decrease in tobramycin susceptibility. Although this in vitro study does not present any data to indicate that this happens in vivo, this is an important finding and one which clinicians should be aware of, as to the potential for this happening in vivo.

Data from our current study expands upon Vecellio et al. and demonstrates how extrapolated peak and trough tobramycin concentrations from less efficient nebulizers can be important drivers in vitro of both bacterial persistence and antimicrobial resistance in CF PA organisms. Classically, poor antimicrobial stewardship, poor treatment adherence and sub-optimal pharmacokinetic/pharmacodynamics (PK/PD) parameters are well associated with driving antibiotic
resistance. This study shows how nebulizer choice can be an important custodian of PA eradication, as well as antimicrobial stewardship. This is an important clinical consideration to maximize organism eradication and minimize antibiotic resistance development.

CONCLUSIONS

This in vitro study emulating standard cycles of ON/OFF inhaled antipseudomonal tobramycin therapy showed that suboptimal concentrations of tobramycin drove increased antibiotic resistance. Extrapolated tobramycin trough concentrations derived from less efficient nebulisers drove antibiotic resistance development by (i) initially allowing for the survival of intermediate and resistant organisms, because nebulizer performance did not achieve critical MIC concentrations sufficient to eradicate the organism, (ii) allowing the development of resistance in those cells that were able to survive the initial tobramycin challenge. For people with CF on TIS treatment, it is important that clinicians and individuals employ a nebulizer that helps mitigate the possibility of an upward drift in antibiotic resistance, thereby protecting the clinical value of TIS within CF care.
ACKNOWLEDGEMENTS

This study was funded by PARI Respiratory Equipment Inc. USA.

CONFLICT OF INTEREST STATEMENT

This paper is for informational purposes only. The authors do not make any recommendations on treatments, devices or pharmacological preparations.

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REFERENCES


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LEGENDS TO FIGURES:

Figure 1: Factors responsible for suboptimal concentration of tobramycin in the CF airways

Figure 2: Sub-inhibitory concentrations of tobramycin- a driver of persistence and antibiotic resistance in *Pseudomonas aeruginosa* isolates from individuals with CF

Figure 3: Description of *in vitro* tobramycin cycling regimens employed

Figure 4a: Bactericidal activity (%) of extrapolated nebulizer peak and trough tobramycin concentrations against first isolates of *Pseudomonas aeruginosa* (N=32)

Figure 4b: Bactericidal activity (%) of extrapolated nebulizer peak and trough tobramycin concentrations against chronic isolates of *Pseudomonas aeruginosa* (N=82)

Figure 5a: Antibiotic susceptibility of SENSITIVE isolates following 28 days ON Tobramycin

Figure 5b: Antibiotic susceptibility of INTERMEDIATE isolates following 28 days ON Tobramycin

Figure 5c: Antibiotic susceptibility of RESISTANT isolates following 28 days ON Tobramycin

Figure 6a: Antibiotic susceptibility of SENSITIVE isolates following 28 days ON Tobramycin & 28 days OFF Tobramycin

Figure 6b: Antibiotic susceptibility of INTERMEDIATE isolates following 28 days ON Tobramycin & 28 days OFF Tobramycin

Figure 6c: Antibiotic susceptibility of RESISTANT isolates following 28 days ON Tobramycin & 28 days OFF Tobramycin

Figure 7a: Antibiotic susceptibility of SENSITIVE isolates following 28 days ON Tobramycin & 28 days ON Tobramycin
Figure 7b: Antibiotic susceptibility of INTERMEDIATE isolates following 28 days ON Tobramycin & 28 days ON Tobramycin

Figure 7c: Antibiotic susceptibility of RESISTANT isolates following 28 days ON Tobramycin & 28 days ON Tobramycin

LEGEND TO TABLE:

Table 1: Published and extrapolated Peak/Trough tobramycin concentrations employed
Figure 1: Factors responsible for suboptimal concentration of tobramycin in the CF airways.

Tobramycin concentrations in the airways are highly variable due mainly to several components, including:

(i) patient-related factors:
- breathing pattern,
- airway anatomy,
- age,
- sputum production/volume,
- airway obstruction,
- degree of mucus plugging
- disease severity,

(ii) patient-compliance with nebulized tobramycin therapy
- previous adherence to nebuliser therapy, as a percentage of the prescribed regimen, has been reported as 65–80% via self-reporting, 50–60% via clinician reporting, and 36% via electronic download.6,7

(iii) the inhibitory effect of sputum constituents which bind aminoglycosides, including:
- mucin,
- glycoprotein
- free eukaryotic and bacterial DNA

(iv) biofilm age, as older biofilms are composed of more resistant bacteria (100–1,000-fold greater) compared to younger biofilms

(v) device-related factors including nebulizer type and efficiency
Sub-inhibitory concentrations of tobramycin- a driver of persistence and antibiotic resistance in Pseudomonas aeruginosa isolates from people with cystic fibrosis

Methods

Sputum
E-test (Tobramycin)

Sub-inhibitory concentrations of tobramycin- a driver of persistence and antibiotic resistance in Pseudomonas aeruginosa isolates from people with cystic fibrosis

Findings

Persistence

First isolates (N=32)
- Absence of tobramycin
- Presence of tobramycin 37°C (28d cycle)

Chronic isolates (N=82)
- Absence of tobramycin
- Presence of tobramycin 37°C (28d cycle)

Resistance

After 28 days ON tobramycin

versus

28 days OFF

Isolates more sensitive to tobramycin following a 28d OFF cycle (p=0.008)

Take Home Messages

It is important that both clinicians advocate and that patients use the nebuliser device which maximises tobramycin delivery and cycle antibiotic use to:- (i) optimise the eradication of first isolates and (ii) help mitigate the development of antibiotic resistance particularly during chronic infection.
Figure 3: Description of the four tobramycin cycling study arms
Figure 4a  Bactericidal activity (%) of extrapolated nebulizer peak and trough tobramycin concentrations against first isolates of *Pseudomonas aeruginosa* (N=32)

![Graph showing bactericidal activity of tobramycin concentrations against first isolates of *Pseudomonas aeruginosa*.](image)

Figure 4b  Bactericidal activity (%) of extrapolated nebulizer peak and trough tobramycin concentrations against chronic isolates of *Pseudomonas aeruginosa* (N=82)

![Graph showing bactericidal activity of tobramycin concentrations against chronic isolates of *Pseudomonas aeruginosa*.](image)
Figure 5a  Antibiotic susceptibility of SENSITIVE isolates following 28 days ON Tobramycin

Minimum Inhibitory Concentration to Tobramycin (MIC) [mg/L] vs Concentration of Tobramycin (mg/L)

- CONTROL
- 7
- 17

Figure 5b  Antibiotic susceptibility of INTERMEDIATE isolates following 28 days ON Tobramycin

Minimum Inhibitory Concentration to Tobramycin (MIC) [mg/L] vs Concentration of Tobramycin (mg/L)

- CONTROL
- 7
- 17
- 65
- 83
- 196

Figure 5c  Antibiotic susceptibility of RESISTANT isolates following 28 days ON Tobramycin

Minimum Inhibitory Concentration to Tobramycin (MIC) [mg/L] vs Concentration of Tobramycin (mg/L)

- CONTROL
- 7
- 17
- 65
- 83
Figure 6a  Antibiotic susceptibility of SENSITIVE isolates following 28 days ON Tobramycin & 28 days OFF Tobramycin

Figure 6b  Antibiotic susceptibility of INTERMEDIATE isolates following 28 days ON Tobramycin & 28 days OFF Tobramycin

Figure 6c  Antibiotic susceptibility of RESISTANT isolates following 28 days ON Tobramycin & 28 days OFF Tobramycin
Figure 7a  Antibiotic susceptibility of SENSITIVE isolates following 28 days ON Tobramycin & 28 days ON Tobramycin

Figure 7b  Antibiotic susceptibility of INTERMEDIATE isolates following 28 days ON Tobramycin & 28 days ON Tobramycin

Figure 7c  Antibiotic susceptibility of RESISTANT isolates following 28 days ON Tobramycin & 28 days ON Tobramycin
Table 1:

<table>
<thead>
<tr>
<th>Nebulizer</th>
<th>Peak Tobramycin Concentration (μg/g)</th>
<th>Trough Tobramycin Concentration (μg/g)</th>
<th>Fine Particle Mass (mg)¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>PARI LC PLUS</td>
<td>762*</td>
<td>65*</td>
<td>74 ± 6</td>
</tr>
<tr>
<td>Misty Neb 2035G</td>
<td>196**</td>
<td>17**</td>
<td>19 ± 2</td>
</tr>
<tr>
<td>Sidestream 12-NEB400</td>
<td>83**</td>
<td>7**</td>
<td>8 ± 7</td>
</tr>
</tbody>
</table>

* Taken from Hubert et al ¹² (Peak tobramycin concentration ranged from 754-769 μg/g)
** Extrapolated from Vecellio et al¹