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Resistance suppression by high-intensity, short-duration aminoglycoside exposure against hypermutable and non-hypermutable *Pseudomonas aeruginosa*

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Objectives: Hypermutable bacteria are causing a drastic problem via their enhanced ability to become resistant. Our objectives were to compare bacterial killing and resistance emergence between differently shaped tobramycin concentration–time profiles at a given *f*AUC/MIC, and determine the tobramycin exposure durations that prevent resistance.

Methods: Static concentration time–kill studies over 24 h used *Pseudomonas aeruginosa* WT strains (ATCC 27853 and PAO1) and hypermutable PAOΔ*mutS*. *f*AUC/MIC values of 36, 72 and 168 were assessed at initial inocula of 10^6 and 10^4 cfu/mL (all strains) and $10^{1.2}$ cfu/mL (PAOΔ*mutS* only) in duplicate. Tobramycin was added at 0 h and removed at 1, 4, 10 or 24 h. Proportions of resistant bacteria and MICs were determined at 24 h. Mechanism-based modelling was conducted.

Results: For all strains, high tobramycin concentrations over 1 and 4 h resulted in more rapid and extensive initial killing compared with 10 and 24 h exposures at a given *f*AUC/MIC. No resistance emerged for 1 and 4 h durations of exposure, although extensive regrowth of susceptible bacteria occurred. The 24 h duration of exposure revealed less regrowth, but tobramycin-resistant populations had completely replaced susceptible bacteria by 24 h for the 10^6 cfu/mL inoculum. The hypermutable PAOΔ*mutS* showed the highest numbers of resistant bacteria. Total and resistant bacterial counts were described well by novel mechanism-based modelling.

Conclusions: Extensive resistance emerged for 10 and 24 h durations of exposure, but not for shorter durations. The tobramycin concentration–time profile shape is vital for resistance prevention and should aid the introduction of optimized combination regimens.

Introduction

A global healthcare crisis is arising from Gram-negative bacteria, such as *Pseudomonas aeruginosa*, where effective antibiotics are increasingly scarce leading to difficult-to-treat life-threatening infections.[1–4] The occurrence of hypermutator phenotypes in *P. aeruginosa* clinical isolates is exacerbating the problem because of their enhanced ability to become resistant.[5,6] The fast-acting aminoglycoside tobramycin causes significant bacterial killing although extensive resistance may occur following monotherapy.[7] Tobramycin is a protein synthesis inhibitor[8] that has also been found to disrupt the bacterial outer membrane.[9,10] The most important resistance mechanisms of *P. aeruginosa* against aminoglycosides are the up-regulation of the MexXY-OprM efflux pump,[11–13] reduced permeability of the outer membrane, and enzymes that inactivate aminoglycosides intracellularly by phosphorylation, acetylation or adenylation.[14]

Hypermutable strains of *P. aeruginosa* are of major concern, particularly in chronic respiratory infections such as those in patients with cystic fibrosis (CF).[15–17] Hypermutation is caused by defects in DNA or error repair systems[18] and is most commonly due to mutations in the *mutS* gene, encoding a component of the

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methyl-directed mismatch repair system.[5,19,20] It provides bacteria with an advantage through the ability to adapt quickly to stressful and fluctuating environments via rapidly gaining or enhancing resistance mechanisms.[15,21,22]

Two pharmacokinetic/pharmacodynamic indices are most commonly used as predictors for bacterial killing by aminoglycosides, being the *f*AUC/MIC and the fC_{max}/MIC.[23–25] The fC_{max}/MIC relies on the concentration at a single timepoint within a dosage interval. The *f*AUC/MIC only considers the total (i.e. time-averaged) exposure across a 24 h period and suggests the same extent of bacterial killing regardless of the shape of the concentration–time profile. Once- and thrice-daily intravenous aminoglycoside therapy at the same daily dose were found to be equally effective clinically against pulmonary exacerbations of CF, but the emergence of resistance was not studied.[26,27] However, in a small clinical study in 33 patients with CF, tobramycin C_{max}/MIC was found as the best predictor of clinical outcome as measured by lung function;[28] the investigators suggested that resistance was greater after once-daily dosing, but an alternative analysis may have led to a different conclusion. Recently we have shown that high ciprofloxacin concentrations for a short duration of exposure (at a specific *f*AUC/MIC) resulted in resistance prevention.[29]

The main objective of the current investigation was to evaluate the bacterial killing and emergence of resistance resulting from differently shaped tobramycin concentration–time profiles at a given *f*AUC/MIC. We aimed to determine whether short-duration, high concentrations were more efficient in bacterial killing and prevention of the emergence of resistance compared with low concentrations over longer durations. Furthermore, we sought to evaluate resistance prevention in the worst-case scenario of a hypermutable strain. To address these objectives we used *in vitro* time–kill studies to assess bacterial killing and resistance emergence for different concentration–time profiles, at given *f*AUC/MIC in hypermutable and non-hypermutable *P. aeruginosa*.

Materials and methods

Bacterial strains and media

The *P. aeruginosa* ATCC 27853 and PAO1 WT reference strains were used in this study. We also used the isogenic hypermutable *P. aeruginosa* PAOΔ*mutS* strain that was constructed from the PAO1 WT reference strain by Mena *et al*.[30] via deletion of the *mutS* gene. All susceptibility and time–kill studies were performed in CAMHB (containing 20–25 mg/L Ca^{2+} and 10–12.5 mg/L Mg^{2+}; BD, Sparks, MD, USA). Viable counting was performed on cation-adjusted Mueller–Hinton agar (containing 25 mg/L Ca^{2+} and 12.5 mg/L Mg^{2+}; Medium Preparation Unit, The University of Melbourne, Parkville, Victoria, Australia). Drug-containing agar plates were prepared using cation-adjusted Mueller–Hinton agar (BD) supplemented with the appropriate amount of tobramycin (AK Scientific, Union City, MD, USA). The antibiotic stock solution was prepared in Milli-Q water and subsequently filter-sterilized using a 0.22 μm PVDF syringe filter (Merck Millipore, Cork, Ireland).

Time–kill experiments

To assess bacterial killing and emergence of resistance, time–kill experiments were performed in duplicate as previously described[29] for the different tobramycin exposure profiles. We studied three overall (i.e. time-averaged) tobramycin exposures, corresponding to *f*AUC/MIC of 36, 72 and 168; two exposures were above and one below the recommended *f*AUC/MIC exposure of 42 for the bactericidal effect.[24] For MICs ≤1 mg/L (83% of *P. aeruginosa* isolates reported by EUCAST),[31] the two lower exposures are achievable at common clinical doses, whereas *f*AUC/MIC of 168 requires a high clinical dose in ICU patients.[32] The tobramycin agar dilution MIC using the CLSI method[33] was 0.5 mg/L for PAO1 and ATCC 27853; while for PAOΔ*mutS* it was 1 mg/L due to the resistant bacterial subpopulations.[34]

Table 1. Tobramycin concentrations (mg/L) and durations of exposure for each studied *f*AUC/MIC against three strains, ATCC 27853 (MIC 0.5 mg/L), PAO1 (MIC 0.5 mg/L) and PAOΔ*mutS* (MIC 1 mg/L)

Drug exposure duration (h)	*f*AUC/ MIC: 36	*f*AUC/ MIC: 72	*f*AUC/ MIC: 168
ATCC 27853 and PAO1 control	0	0	0
1	18	36	84
4	4.5	9	21
10	1.8	3.6	8.4
24	0.75	1.5	3.5
PAOΔ*mutS* control	0	0	0
1	36	72	168
4	9	18	42
10	3.6	7.2	16.8
24	1.5	3	7

Overall exposures were achieved by exposing the bacteria to appropriate tobramycin concentrations for the durations of 1, 4, 10 and 24 h, as reported in Table 1. Tobramycin was dosed at 0 h and rapidly removed at the respective timepoint via two or three sequential centrifugation and resuspension steps, as we previously described.[29] This method assured that the tobramycin concentrations were negligible (<0.16× MIC) after drug removal. These different exposures were studied at initial inocula of 10^6 and 10^4 cfu/mL (all three strains), as well as $10^{1.2}$ cfu/mL (PAOΔ*mutS* only). The probability of at least one pre-existing bacterial cell that was resistant to 2.5 mg/L tobramycin was ≤7.1% at 10^4 cfu/mL for ATCC 27853 and PAO1, and 2.3% at $10^{1.2}$ cfu/mL for PAOΔ*mutS*. In contrast, for all strains at 10^6 cfu/mL and PAOΔ*mutS* at 10^4 cfu/mL, this probability was >96.7%. All studies included a growth control. Viability counts of bacteria as described previously[29] were determined within 5 min prior to dosing and at 0.5, 2, 6, 10 (or 12) and 24 h after dosing as well as 5 min before and 10 min after drug removal to confirm minimal loss of bacteria via the drug removal procedure.

Emergence of resistance

The proportion of resistant bacteria (PRB) and MICs were determined at 0 (i.e. before treatment) and 24 h. Tobramycin was removed before evaluating emergence of resistance. Agar dilution MICs were determined once the bacterial suspensions were spectrophotometrically adjusted (i.e. dilution in fresh, pre-warmed, sterile CAMHB) to an inoculum of 10^6 cfu/mL, unless the suspension was already below this inoculum. Agar plates containing 1.25 mg/L (PAO1), 1.5 mg/L (ATCC 27853), 2.5 mg/L (all three strains) and 5 mg/L (PAOΔ*mutS*) tobramycin were used for determining the PRB. Antibiotic-containing agar plates were incubated for 3 days and the $\log_{10}$ PRB was calculated as the difference, on $\log_{10}$ scale, between the viability of resistant bacteria on antibiotic-containing agar plates and total population viability on drug-free plates.

Some of the viable counts at 24 h were too low to quantify colonies on antibiotic-containing agar plates. These bacterial suspensions still

provided information on the upper limit of the $\log_{10}$ PRB (e.g. $\log_{10}$ PRB ≤ -6). To include these data we used the following reporting rules. If the PRB was not quantifiable, but the upper limit was within 1 $\log_{10}$ of the PRB for the growth control, we assumed the PRB was unchanged and used the value of the growth control. If the PRB was not quantifiable and the upper limit was >1 $\log_{10}$ higher than the PRB for the growth control, the PRB of this treatment was reported as missing.

Mechanism-based modelling of bacterial killing and resistance

Mechanism-based pharmacokinetic/pharmacodynamic models were developed to characterize the time-course of bacterial killing and emergence of tobramycin resistance.

Life cycle growth model

The growth and replication of *P. aeruginosa* was described by a life cycle growth model that accounts for the underlying biological processes.[35–37] A diagram of the model structure is shown in Figure 1. Inclusion of three pre-existing bacterial populations (the bacterial populations present prior to treatment)—susceptible, intermediate and resistant—best described the observed data. For each of these populations, the model included two bacterial states: state 1 representing the bacteria preparing for replication and state 2 those immediately before replication;[36–39] e.g. cfu_{S1} denotes the susceptible bacteria in state 1 and cfu_{S2} the susceptible bacteria in state 2.

The total bacterial population (cfu_{all}) was defined as the sum of bacteria in all subpopulations and bacterial states:

$$cfu_{all} = cfu_{S1} + cfu_{S2} + cfu_{I1} + cfu_{I2} + cfu_{R1} + cfu_{R2} \quad (1)$$

where the susceptible population in state 1 (cfu_{S1}) was described by

$$\frac{d(cfu_{S1})}{dt} = ALIVE_S \cdot (REP \cdot k_{21} \cdot cfu_{S2} - k_{12} \cdot cfu_{S1} - KILL_{SPAE1} \cdot cfu_{S1}) \quad (2)$$

and the susceptible population in state 2 (cfu_{S2}) was described by

$$\frac{d(cfu_{S2})}{dt} = ALIVE_S \cdot (-k_{21} \cdot cfu_{S2} + k_{12} \cdot cfu_{S1} - KILL_{SPAE1} \cdot cfu_{S2}) \quad (3)$$

The intermediate (cfu_{I1}, cfu_{I2}) and resistant (cfu_{R1}, cfu_{R2}) populations were modelled similarly.

In Equations (2) and (3), the first-order growth rate constant k_{12} was defined as 60/MGT, with MGT representing the mean generation time for each of the bacterial populations. The first-order replication rate constant k_{21} was set to 50 h^{-1} as described previously.[37] $ALIVE_S$ was 1 while the estimated ($cfu_{S1}+cfu_{S2}$) was $\geq$0.5 cells in the entire broth volume, otherwise $ALIVE_S$ was 0. This part of the model allowed us to describe bacterial eradication. The replication factor REP defines the probability of successful replication, as described previously.[37]

$$REP = 2 \cdot \left(1 - \frac{cfu_{all}}{cfu_{all} + cfu_{max}}\right) \quad (4)$$

REP approaches 2 when the total bacterial count (cfu_{all}) is low, resulting in a 100% probability of successful replication. As cfu_{all} approaches the maximum population size (cfu_{max}), REP approaches 1, which reflects a 50% probability of successful replication and ensures that cfu_{all} does not exceed cfu_{max}. The term $KILL_{SPAE1}$ describes bacterial killing by tobramycin including the post-antibiotic effect (PAE),[40] as described in Equations (5) and (6) below.

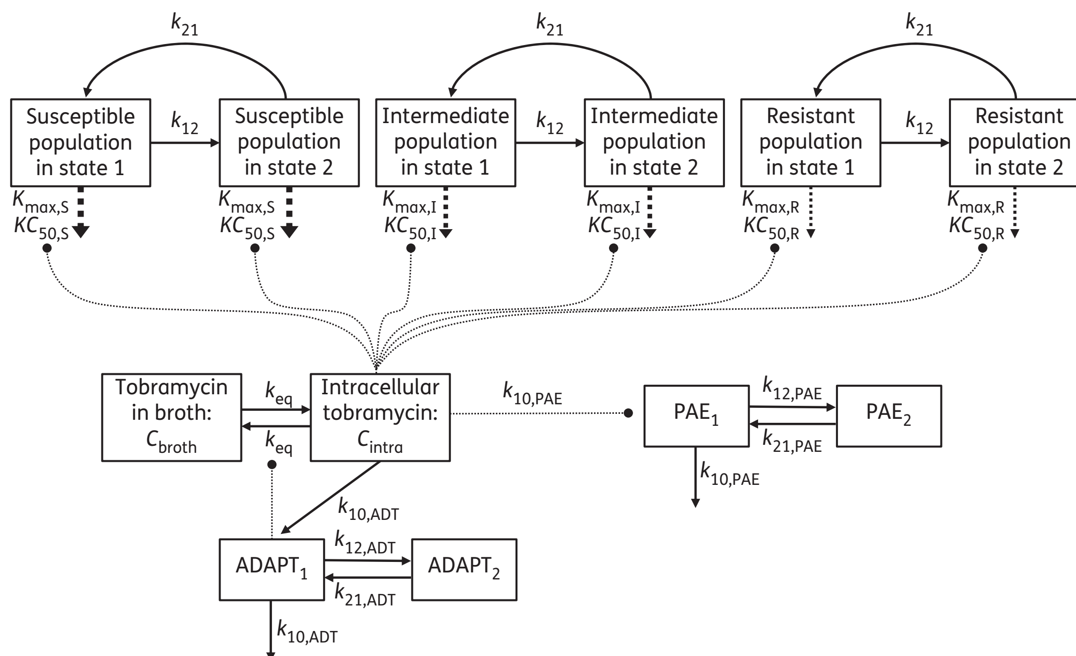

Figure 1. Model diagram of the life cycle growth model including three populations, susceptible, intermediate and resistant, with two states each to describe bacterial replication. The maximum killing rate constants (K_{max}) and the antibiotic concentrations (KC_{50}) causing 50% of K_{max} are explained in Table 2. The concentration of tobramycin in broth (C_{broth}), intracellular tobramycin (C_{intra}), $ADAPT_1$, $ADAPT_2$ and all corresponding rate constants are described in the Materials and methods section.

Bacterial killing by tobramycin including PAE

A prolonged PAE is a trait of tobramycin[40] hence it is represented in the bacterial killing function $KILL_{SPAE1}$ in Equation 5. The $KILL_{SPAE2}$ (Equation 6) is in equilibrium with $KILL_{SPAE1}$ and provides a delay such that bacterial killing occurs for some time after removal of tobramycin from the system.

$$\frac{d(\mathrm{KILL}_{\mathrm{SPAE1}})}{dt} = k_{10,\mathrm{PAE}} \cdot (\mathrm{KILL}_{\mathrm{S}} - \mathrm{KILL}_{\mathrm{SPAE1}}) - k_{12,\mathrm{PAE}} \cdot \mathrm{KILL}_{\mathrm{SPAE1}} + k_{21,\mathrm{PAE}} \cdot \mathrm{KILL}_{\mathrm{SPAE2}} \quad (5)$$

$$\frac{d(\mathrm{KILL}_{\mathrm{SPAE2}})}{dt} = k_{12,\mathrm{PAE}} \cdot \mathrm{KILL}_{\mathrm{SPAE1}} - k_{21,\mathrm{PAE}} \cdot \mathrm{KILL}_{\mathrm{SPAE2}} \quad (6)$$

The PAE rate constants $k_{10,PAE}$, $k_{12,PAE}$ and $k_{21,PAE}$ represent the delayed killing that results from the PAE and were defined as $k_{10,PAE}=1/MTT_{10,PAE}$, $k_{12,PAE}=1/MTT_{12,PAE}$ and $k_{21,PAE}=1/MTT_{21,PAE}$, where MTT denotes mean turnover time. Modelling the PAO1 did not require this PAE.

The bacterial killing by tobramycin is represented by $KILL_S$:

$$\mathrm{KILL}_{\mathrm{S}} = \frac{K_{\mathrm{max,S}} \cdot C_{\mathrm{intra}}}{C_{\mathrm{intra}} + KC_{50,\mathrm{S}}} \quad (7)$$

where C_{intra} is the (estimated) intracellular tobramycin concentration. $K_{max,S}$ is the maximum killing rate constant and $KC_{50,S}$ is the C_{intra} required to achieve 50% of $K_{max,S}$.[9] Bacterial killing and PAE of the I and R populations were described similarly. The C_{intra} is defined in Equation (8), as described previously.[9]

$$\frac{d(C_{\mathrm{intra}})}{dt} = k_{\mathrm{eq}} \cdot C_{\mathrm{broth}} - k_{\mathrm{eq}} \cdot (\mathrm{ADAPT}_1 + 1) \cdot C_{\mathrm{intra}} \quad (8)$$

The rate constant k_{eq} is defined as $\ln(2)/t_{1/2eq}$, where $t_{1/2eq}$ is the equilibration half-life between tobramycin in broth and the intracellular space. The term $ADAPT_1$ reflects adaptive resistance of *P. aeruginosa* against tobramycin. An increase in adaptive resistance ($ADAPT_1$, defined below) decreases C_{intra} and thereby reduces the extent of bacterial killing by tobramycin.

Adaptive resistance to tobramycin

Incorporating adaptive resistance in the mechanism-based model was required to describe the observed bacterial count profiles in our experiments. Adaptive resistance against aminoglycosides is often caused by the overexpression of the MexXY-OprM efflux pump[41] or inhibition of energy-dependent uptake in *P. aeruginosa*,[42] both of which would decrease C_{intra}, as described above. It was described by a two-compartment model for $ADAPT_1$ (Equation 9) and $ADAPT_2$ (Equation 10)[9]:

$$\frac{d(\mathrm{ADAPT}_1)}{dt} = k_{10,\mathrm{ADT}} \cdot \left(\frac{\mathrm{ADAPT}_{\mathrm{max}} \cdot C_{\mathrm{intra}}}{C_{\mathrm{intra}} + \mathrm{EC}_{50,\mathrm{ADT}}} - \mathrm{ADAPT}_1\right) - k_{12,\mathrm{ADT}} \cdot \mathrm{ADAPT}_1 + k_{21,\mathrm{ADT}} \cdot \mathrm{ADAPT}_2 \quad (9)$$

$$\frac{d(\mathrm{ADAPT}_2)}{dt} = k_{12,\mathrm{ADT}} \cdot \mathrm{ADAPT}_1 - k_{21,\mathrm{ADT}} \cdot \mathrm{ADAPT}_2 \quad (10)$$

where $ADAPT_{max}$ is the maximum extent of adaptive resistance and $EC_{50,ADT}$ is the C_{intra} that induces the half-maximum extent of adaptive resistance. The peripheral adaptation compartment $ADAPT_2$ allows for the delayed decline of adaptive resistance after removal of tobramycin. The adaptive resistance rate constants $k_{10,ADT}$, $k_{12,ADT}$ and $k_{21,ADT}$ describe the time-course of adaptive resistance and are defined as $k_{10,ADT}=1/MTT_{10,ADT}$, $k_{12,ADT}=1/MTT_{12,ADT}$ and $k_{21,ADT}=1/MTT_{21,ADT}$.

Resistant bacterial populations on antibiotic-containing agar plates

The viable counts on tobramycin-containing agar plates were modelled simultaneously with the total viable counts on drug-free agar plates. The fractions of subpopulations (susceptible, intermediate, resistant) that were able to grow on tobramycin-containing agar plates at different concentrations were estimated as described previously.[43]

Initial conditions

The total initial inocula ($\log_{10}cfu_{0,4}$, $\log_{10}cfu_{0,6}$) and the PRB for the intermediate ($\log_{10}PRB_I$) and resistant ($\log_{10}PRB_R$) populations were estimated (Table 2). The initial condition for the susceptible population was calculated by subtracting the initial conditions of the intermediate and the resistant populations from the respective total inoculum. All bacteria were initialized in state 1 and the initial conditions for cfu_{S2}, cfu_{I2} and cfu_{R2} were set to 0. $ALIVE_S$, $ALIVE_I$ and $ALIVE_R$ were initialized at 1 and all other equations at 0.

Observation model

The $\log_{10}$ viability counts were fitted using an additive residual error model on $\log_{10}$ scale. A previously described residual error model was utilized to fit directly the number of colonies on a plate when there were less than two colonies per plate observed.[44] Viable counts below the limit of counting and model predictions <0 $\log_{10}$ cfu/mL were plotted as 0.

Estimation

The model parameters were estimated simultaneously using the viable counts on drug-free and tobramycin-containing plates for each of the strains at the 10^4 and 10^6 cfu/mL inocula using the importance sampling algorithm (pmethod=4) in parallelized S-ADAPT (version 1.57), facilitated by SADAPT-TRAN.[45] A coefficient of variation of 15% during the end of estimation allowed the between-curve variability of the parameters to be fixed.[44] The objective function, standard diagnostic plots, plausibility of the parameter estimates and visual predictive checks were utilized to assess competing models.

Results and discussion

In the present study, we demonstrated that different shapes of the concentration–time profile had an important impact on bacterial regrowth and emergence of resistance in *P. aeruginosa* ATCC 27853, PAO1 and PAOΔ*mutS*. The $\log_{10}$ PRB for ATCC 27853 was −5.36 on 1.5 mg/L tobramycin plates and −6.43 on 2.5 mg/L tobramycin plates before treatment. PAO1 had a $\log_{10}$ PRB of −5.34 on 1.25 mg/L and −6.77 on 2.5 mg/L tobramycin plates before treatment. The hypermutable PAOΔ*mutS* had a $\log_{10}$ PRB before treatment of −4.14 on 2.5 mg/L and −5.62 on 5 mg/L tobramycin plates. These results show a dramatic difference in PRB before treatment observed between the PAOΔ*mutS* and the two WT strains (ATCC 27853 and PAO1) on 2.5 mg/L tobramycin plates, which was expected due to the hypermutable strain having an increased likelihood of mutating.

The extent of initial killing of *P. aeruginosa* increased with tobramycin exposure (*f*AUC/MIC) for all three strains, as expected for this fast-acting aminoglycoside. At a given *f*AUC/MIC, the extent of initial bacterial killing increased with concentration as the duration of exposure became shorter (Figure 2). In general, at a given *f*AUC/MIC, the high concentrations for short durations

Table 2. Population parameter estimates for tobramycin against three strains of *P. aeruginosa*

Parameter	Symbol (unit)	Population estimate (SE%) for the strain		
		ATCC 27853	PAO1	PAOΔ*mutS*
Bacterial growth and subpopulations				
$\log_{10}$ initial inoculum				
10^4 cfu/mL	$\log_{10}cfu_{0,4}$	4.20 (4.32)	4.23 (1.49)	4.50 (1.97)
10^6 cfu/mL	$\log_{10}cfu_{0,6}$	5.94 (2.84)	5.56 (1.78)	6.05 (1.82)
MGT				
susceptible population	MGT_S (min)	87.3 (7.24)	41.6 (1.86)[a]	63.9 (8.67)
intermediate population	MGT_I (min)	49.3 (3.59)	—	53.5 (3.55)
resistant population	MGT_R (min)	79.9 (8.89)	—	82.2 (6.75)
$\log_{10}$ maximum population size	$\log_{10}cfu_{max}$	9.09 (2.25)	8.95 (1.25)	9.16 (1.66)
$\log_{10}$ PRB				
intermediate population	$\log_{10}PRB_I$	−4.25 (3.47)	−4.58 (2.43)	−4.12 (3.79)
resistant population	$\log_{10}PRB_R$	−7.17 (2.73)	−6.39 (1.11)	−5.60 (4.64)
Bacterial killing by tobramycin				
equilibrium half-life between tobramycin in broth and the intracellular space	$t_{1/2eq}$ (min)	25.0, fixed	40.0 (3.17)	25.0, fixed
maximum killing rate constant				
susceptible population	$K_{max,S}$ (h^{-1})	78.9 (13.6)	30.3 (3.25)	24.8 (9.91)
intermediate population	$K_{max,I}$ (h^{-1})	2.23 (20.9)	2.07 (4.64)	2.02 (13.1)
resistant population	$K_{max,R}$ (h^{-1})	14.2 (12.0)	7.38 (5.10)	7.93 (10.3)
intracellular tobramycin concentration causing 50% of K_{max}				
susceptible population	$KC_{50,S}$ (mg/L)	0.154 (15.4)	0.0206 (15.7)	0.0410 (19.6)
intermediate population	$KC_{50,I}$ (mg/L)	0.0295 (23.9)	0.0270 (31.6)	0.115 (31.9)
resistant population	$KC_{50,R}$ (mg/L)	2.89 (26.4)	0.574 (8.03)	2.79 (15.3)
Adaptive resistance				
maximum extent of stimulation of adaptive resistance	$ADAPT_{max}$	13.5 (20.5)	14.2 (4.49)	11.2 (28.1)
intracellular tobramycin concentration causing 50% of $ADAPT_{max}$	$EC_{50,ADT}$ (mg/L)	6.06 (51.9)	11.1 (4.82)	28.8 (9.33)
MTT				
for adaptive resistance	$MTT_{10,ADT}$ (h)	6.81 (23.2)	7.37 (6.81)	13.5 (9.72)
for distribution from the central to the peripheral adaptive compartment	$MTT_{12,ADT}$ (h)	0.917 (50.6)	0.972 (10.3)	0.439 (32.2)
for distribution from the peripheral to the central adaptive compartment	$MTT_{21,ADT}$ (h)	6.0, fixed	6.0, fixed	6.0, fixed
PAE				
MTT				
for the PAE	$MTT_{10,PAE}$ (h)	0.0166 (56.9)	—	0.0158 (29.2)
between the central and peripheral PAE compartment	$MTT_{12,PAE}$ (h)	0.519 (43.3)	—	1.06 (22.0)
between the peripheral and central PAE compartment	$MTT_{21,PAE}$ (h)	0.662 (16.2)	—	0.504 (12.6)
Residual variability				
SD of additive residual error on $\log_{10}$ scale for the				
total population	SD_{cfu}	0.302	0.455	0.459
population on 2.5 mg/L tobramycin plates	SD_{cfu3}	0.137	1.51	0.195
population on 5 mg/L tobramycin plates	SD_{cfu5}	0.307	1.79	0.399

[a]Model only contains one MGT.

of exposure resulted in more extensive regrowth than exposure to lower concentrations over a longer time (Figure 2). It is of great concern that for all strains even the high *f*AUC/MIC of 168, i.e. four times the suggested *f*AUC/MIC breakpoint of 42 for bactericidal effect,[24] did not inhibit the regrowth of bacteria at the initial inoculum of 10^6 cfu/mL. An *f*AUC/MIC of 168 would be expected to be achieved in patients for MICs up to ~0.5 mg/L following a tobramycin dose of 5–6 mg/kg in critically and non-critically ill patients[32,46] and 8–11 mg/kg in patients with CF (both based on a 70 kg patient).[47,48] However, according to EUCAST, 46% of the 25 002 evaluated isolates had an MIC ≥1 mg/L.[31]

Generally, with short durations of exposure (1 and 4 h) the ATCC 27853 strain displayed rapid initial killing of 4–6 $\log_{10}$ that was followed by extensive regrowth after drug removal, whilst the longer durations of exposure (10 and 24 h) had limited or no regrowth (Figure 2a). An exception was the low *f*AUC/MIC of 36 where the longer durations of exposure revealed 4 $\log_{10}$ regrowth. In addition, at the high *f*AUC/MIC of 168 the 4 h

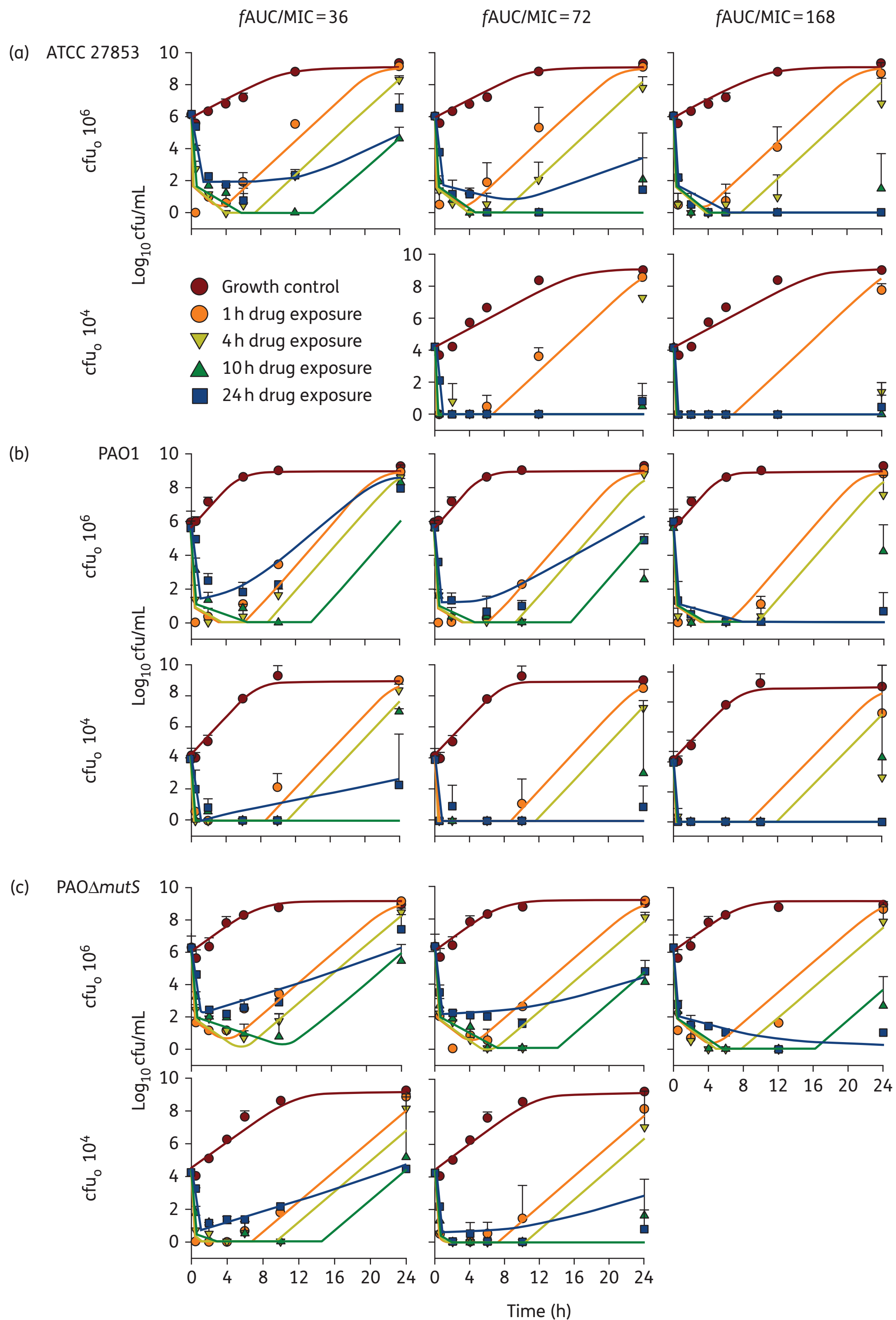

Figure 2. Observed viable counts (mean ± SD) and population predicted profiles (continuous lines in corresponding colours) for *P. aeruginosa* ATCC 27853 (a), PAO1 (b) and PAOΔ*mutS* (c) exposed to tobramycin at an *f*AUC/MIC of 36 (left column), 72 (middle column) and 168 (right column) delivered over 1, 4, 10 or 24 h durations of exposure, at initial inocula (cfu$_o$) of 10^6 and 10^4 cfu/mL, excluding *f*AUC/MIC of 36 for ATCC 27853 and *f*AUC/MIC of 168 for PAOΔ*mutS* for cfu$_o$ 10^4 cfu/mL. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

duration of exposure showed limited regrowth at the 10^4 cfu/mL initial inoculum that likely lacked pre-existing resistant bacteria (Figure 2a). The PAO1 strain showed similar results to the ATCC 27853 strain, with similar extents of initial killing, and mostly the shorter durations of exposure leading to extensive regrowth (Figure 2b). However, the PAO1 strain showed more extensive regrowth for the longer durations of exposure at an *f*AUC/MIC of 36 in comparison with ATCC 27853; extensive regrowth was observed for the 10 and 24 h durations of exposure for PAO1 at the 10^6 cfu/mL inoculum (Figure 2b). The initial killing and bacterial regrowth of PAOΔ*mutS* was mostly comparable to its parental strain PAO1 and ATCC 27853 (Figure 2). At the very low initial inoculum of $10^{1.2}$ cfu/mL complete eradication at 24 h was observed for all tobramycin exposures against PAOΔ*mutS*, except for the 1 h duration of exposure at *f*AUC/MIC of 36 where there was ~2 $\log_{10}$ regrowth (data not shown).

Bacterial regrowth was a common feature of the time–kill profiles for each strain (Figure 2). Notably, at the 10^6 cfu/mL inoculum, although the shorter durations of exposure (1 and 4 h) had more extensive regrowth, their PRB at 24 h were comparable to the growth control for all three strains, suggesting that this regrowth was a susceptible population (Figure 3). Consideration should be given to combination antibiotic therapy as an option for fighting this susceptible regrowth, i.e. using a second antibiotic to prevent the regrowth of the tobramycin-susceptible bacteria. In contrast, the longer durations of exposure (10 and 24 h) at the 10^6 cfu/mL inoculum frequently resulted in increased PRB at 24 h for all three strains at an *f*AUC/MIC of 36 and 72 (Figure 3). These increases in PRB were mostly supported by raised MIC at 24 h for the 10 and 24 h durations of exposure (Table 3). Overall, the hypermutable PAOΔ*mutS* strain had considerably higher PRB values compared with the two WT strains (Figure 3).

For ATCC 27853 and PAO1 the increase in PRB at longer durations could be prevented or considerably limited by the high *f*AUC/MIC of 168 at the 10^6 cfu/mL inoculum, except for the PAO1 at the 24 h duration of exposure. In contrast, for PAOΔ*mutS* essentially the whole bacterial population was replaced by resistant bacteria even at the *f*AUC/MIC of 168. This difference was observed despite the same probability of >96.7% of at least one pre-existing resistant bacterial cell for all three strains. Thus our study demonstrates that resistance suppression is more challenging for the hypermutable strain compared with the two non-hypermutable strains, which is supported by literature reporting that hypermutation results in more difficult-to-eradicate infections.[17,21]

For the 10^4 cfu/mL inoculum, ATCC 27853 and PAO1 exposed to tobramycin for short durations (1 and 4 h) had limited to no increase in PRB and for ATCC 27853 the PRB was unquantifiable for long durations of exposure (Figure 3). These results were supported by the MIC at 24 h remaining unchanged for the 1 and 4 h durations of exposure (Table 3). However, for PAO1 the PRB slightly increased for the 10 and 24 h durations of exposure for the low *f*AUC/MIC of 36 (Figure 3); the 10 h duration of exposure was supported by a raised MIC (Table 3). Similarly, the PAOΔ*mutS* at 10^4 cfu/mL inoculum revealed increases in PRB for the 10 h duration of exposure at an *f*AUC/MIC of 36 and the 24 h duration of exposure at an *f*AUC/MIC of both 36 and 72 (Figure 3). The low inoculum of $10^{1.2}$ cfu/mL (~300 bacteria in 20 mL) was required to minimize the probability of pre-existing resistant bacteria for the hypermutable PAOΔ*mutS* strain. No PRB could be determined for this inoculum due to extensive bacterial killing and the very low numbers of bacteria present (data not shown). Although adaptive resistance mechanisms only require tobramycin exposure[49] these may have been prevented by the extremely fast bacterial killing seen at the $10^{1.2}$ cfu/mL inoculum.

The pre-existing resistant bacteria in the initial inoculum may have been playing a role in the emergence of resistance at the 10^6 cfu/mL inoculum for all strains and at 10^4 cfu/mL for PAOΔ*mutS*. Treatment failure in *P. aeruginosa* infections often occurs from selection of resistant bacteria, e.g. due to overexpression of the MexXY-OprM efflux pump, aminoglycoside-modifying enzymes and decreased outer membrane permeability.[11–14] For PAO1 it is likely that additional resistance pathways not involving amplification of pre-existing resistant bacteria may have played a role in resistance emergence at the 10^4 cfu/mL inoculum. The static concentrations with complete removal of tobramycin at the end of each exposure period would have prevented the development of resistance mechanisms that require sub-MIC concentrations to occur. Common mechanisms involved in the resistance observed in PAO1 could be adaptive resistance via the upregulation of the MexXY-OprM efflux pump,[41,50] which is frequently caused by overexpression of the MexY component, or *de novo* mutations during treatment.[41,51,52] Previous studies have found that a resistance mechanism unrelated to this efflux pump has played a role in aminoglycoside resistance for PAOΔ*mutS*.[6] Ultimately, molecular studies would identify these resistance mechanisms.

Our developed mechanism-based model successfully described simultaneously the time-course of viable counts for the total bacterial population and less susceptible bacterial populations growing on tobramycin-containing plates, for all three strains (Figure 2 and Figure S1, available as Supplementary data at *JAC* Online). The model included three bacterial subpopulations, i.e. susceptible, intermediate and resistant (Figure 1). Tobramycin causes bacterial killing via inhibiting protein synthesis,[8] which is driven by the intracellular tobramycin concentration as described in the model.[9] Aminoglycosides can also cause bacterial killing by disrupting the outer membrane;[10,53,54] however, adding a second mechanism of killing was not required to describe our observed data. It has been suggested that the cellular recovery after tobramycin exposure causes a delay in bacterial regrowth, resulting in a PAE that was included in the model.[40,55] Such a PAE may allow the suppression of regrowth of susceptible subpopulations in patients.[56] The PAE duration has been found to be related to the antibacterial concentration,[57,58] the duration of drug exposure and the inoculum size.[59] A longer duration of exposure was found to result in a longer PAE.[40] The ATCC 27853 and PAOΔ*mutS* strains required a PAE to be present in the model, whilst PAO1 was represented by a simplified model that did not require a PAE.

The bacterial regrowth was also well captured by the model with only minor mispredictions of up to ~2.5 $\log_{10}$. Only for 2 of 80 modelled profiles (ATCC 27853 at the *f*AUC/MIC of 72, 4 h duration of exposure and PAO1 at the *f*AUC/MIC of 36, 10 h duration of exposure, 10^4 cfu/mL inoculum) a larger misprediction occurred as the model predicted complete eradication before bacteria were able to regrow (Figure 2). Emergence of resistance as quantified via tobramycin-containing agar plates was adequately represented in the model (Figure S1). The inclusion of adaptive resistance in the model allowed us to consider the likely cause of emergence of aminoglycoside resistance at low initial inocula.[42,60–63] Incorporating a function for adaptive resistance in

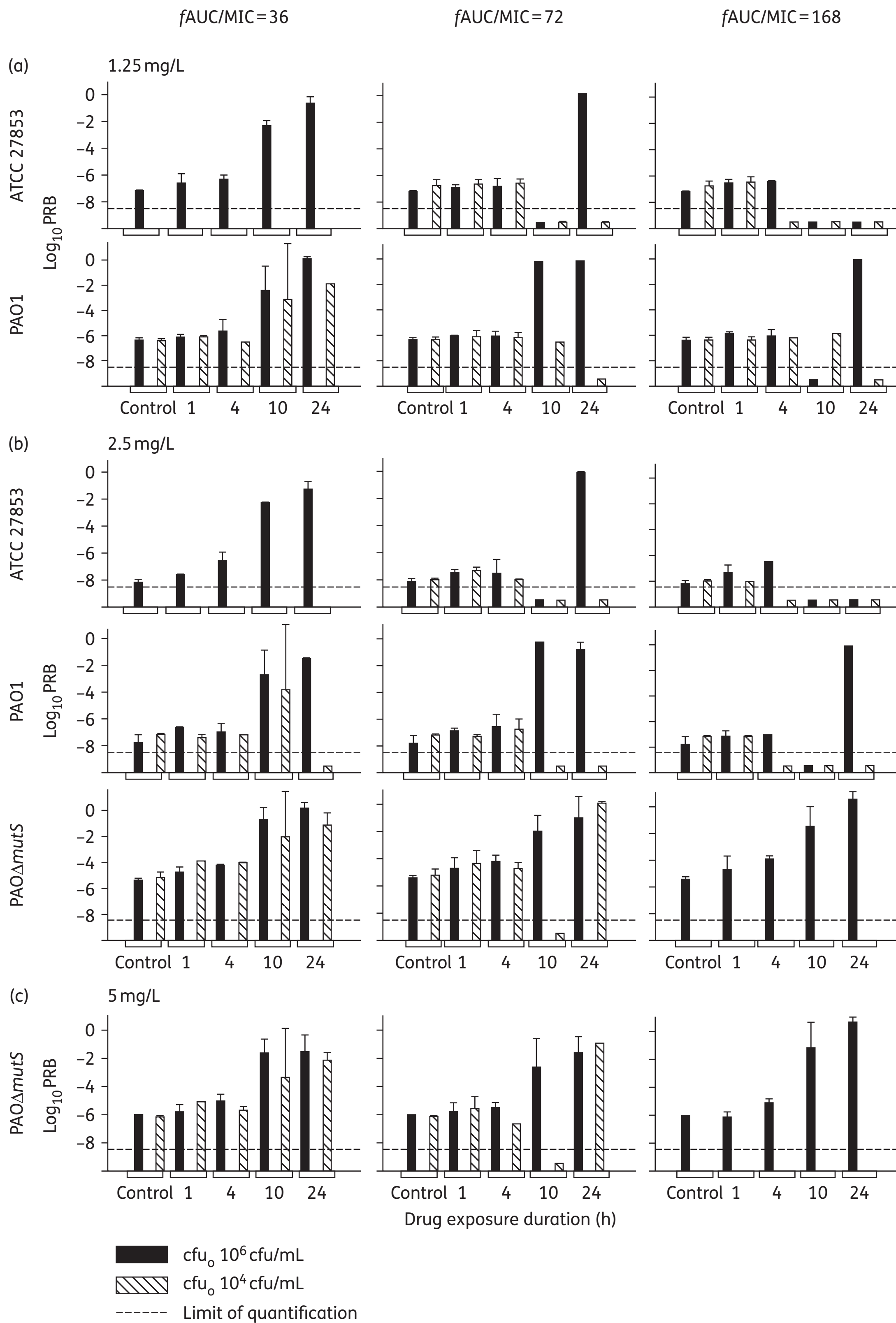

Figure 3. Log_{10} PRB (mean ± SD) at 24 h on agar plates containing 1.25 (1.5 for ATCC 27853; a), 2.5 (b) and 5 (c) mg/L tobramycin. This figure shows *f*AUC/MIC values of 36 (left column), 72 (middle column) and 168 (right column) for the *P. aeruginosa* ATCC 27853, PAO1 and PAOΔ*mutS* delivered over 1, 4, 10 or 24 h durations of exposure, at initial inocula (cfu_o) of 10^6 and 10^4 cfu/mL, excluding *f*AUC/MIC of 36 for ATCC 27853 and *f*AUC/MIC of 168 for PAOΔ*mutS* for cfu_o 10^4 cfu/mL. When a treatment yielded complete eradication or when there were no colonies on antibiotic-containing agar plates no PRB could be determined, and therefore is shown below the limit of quantification as −9.5 log_{10}.

Table 3. MIC (mg/L) at 24 h [geometric mean (range)] for tobramycin *f*AUC/MIC of 36, 72 and 168 delivered over various durations of exposure and initial inocula (cfu_o) against *P. aeruginosa* ATCC 27853 (top), PAO1 (middle) and PAOΔ*mutS* (bottom)

	*f*AUC/MIC: 36		*f*AUC/MIC: 72		*f*AUC/MIC: 168	
Drug exposure duration (h)	cfu_o 1×10^6	cfu_o 1×10^4	cfu_o 1×10^6	cfu_o 1×10^4	cfu_o 1×10^6	cfu_o 1×10^4
ATCC 27853 control	0.5 (0.5–0.5)	ND	0.5 (0.5–0.5)	0.5 (0.5–0.5)	0.5 (0.5–0.5)	0.5 (0.5–0.5)
1	0.5 (0.5–0.5)	ND	0.5 (0.5–0.5)	0.5 (0.5–0.5)	0.5 (0.5–0.5)	0.5 (0.5–0.5)
4	0.5 (0.5–0.5)	ND	0.5 (0.5–0.5)	0.5 (0.5–0.5)	0.5 (0.5–0.5)	0.25
10	1.0 (0.5–2.0)	ND	0.5	—	0.25	—
24	**4.0 (4.0–4.0)**	ND	**4.0**	—	—	—
PAO1 control	0.5 (0.5–0.5)	0.5 (0.5–0.5)	0.5 (0.5–0.5)	0.5 (0.5–0.5)	0.5 (0.5–0.5)	0.5 (0.5–0.5)
1	0.5 (0.5–0.5)	0.5 (0.5–0.5)	0.5 (0.5–0.5)	0.7 (0.5–1.0)	0.7 (0.5–1.0)	0.5 (0.5–0.5)
4	0.7 (0.5–1.0)	0.5 (0.5–0.5)	0.7 (0.5–1.0)	0.7 (0.5–1.0)	0.7 (0.5–1.0)	0.5
10	**2.0 (0.5–8.0)**	**2.0 (0.5–8.0)**	0.25	1.0	0.5 (0.25–1.0)	—
24	**5.7 (4.0–8.0)**	—	**4.0 (4–4)**	—	—	—
PAOΔ*mutS* control	1.0 (1.0–1.0)	1.0 (1.0–1.0)	1.0 (1.0–1.0)	1.0 (1.0–1.0)	1.0 (1.0–1.0)	ND
1	1.0 (1.0–1.0)	2.8 (2.0–4.0)	1.0 (1.0–1.0)	2.0 (2.0–2.0)	1.0 (1.0–1.0)	ND
4	1.0 (1.0–1.0)	2.0 (2.0–2.0)	1.0 (1.0–1.0)	1.0 (1.0–1.0)	1.0 (1.0–1.0)	ND
10	**8.0 (8.0–8.0)**	1.0	0.7 (0.5–1.0)	—	—	ND
24	**11.3 (8.0–16.0)**	**5.7 (4.0–8.0)**	**5.7 (4.0–8.0)**	—	—	ND

ND indicates that this inoculum was not carried out for this strain in this study.
MICs are in bold if they were at least 4-fold above baseline. No range is provided if only one replicate was available. PAOΔ*mutS* at cfu_o of $10^{1.2}$ cfu/mL had MIC 1.0 mg/L with a range 1.0–1.0 mg/L for the growth control, whilst extensive bacterial killing did not allow determination of the MIC for any treated arms.

addition to amplification of pre-existing resistant bacteria was required to describe best the observed data. Overall, the model accurately described the bacterial killing (Figure 2) and emergence of resistance (Figure S1) for all three strains. We recognize that both the *in vitro* experiments and mechanism-based model lack an immune system effect and therefore the results of this study would be most applicable to immunocompromised patients. In addition, the static nature of the concentration delivery for defined durations generated different concentration–time profiles to those that would be observed in patients.

In conclusion, the study allowed us to determine whether different tobramycin concentration–time profiles at a given overall exposure affect not only bacterial killing, but also resistance prevention. Our results for the 24 h duration of exposure demonstrated that, despite limited regrowth, there was complete replacement of susceptible bacteria with tobramycin-resistant populations when regrowth occurred. Emergence of resistance was suppressed for 1 and 4 h durations of exposure supporting once-daily dosing, although extensive regrowth of susceptible bacteria occurred. Therefore, investigation of dosage regimens involving short-duration, high tobramycin concentrations together with a second antibiotic to prevent tobramycin-susceptible regrowth is warranted. Our mechanism-based mathematical model would assist in the optimization of such antibiotic combinations. Combination dosage regimens may be particularly beneficial and are urgently required to combat hypermutable strains arising in patients. Studies in dynamic *in vitro* systems that simulate antibiotic concentration–time profiles as observed in patients are necessary to evaluate such innovative aminoglycoside combination dosage regimens and translate these regimens to benefit patients.

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Transparency declarations

None to declare.

Disclaimer

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Supplementary data

Figure S1 is available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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