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2 **amikacin against *Pseudomonas aeruginosa* in a dynamic *in vitro* hollow-fibre infection**
3 **model.**
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52 **Abstract**

53 Given that aminoglycosides, such as amikacin, may be used for multi-drug resistant
54 *Pseudomonas aeruginosa* infections, optimization of therapy is paramount for improved
55 treatment outcomes. This study aims to investigate the pharmacodynamics of different
56 simulated intravenous amikacin doses on susceptible *P. aeruginosa* to inform ventilator-
57 associated pneumonia and sepsis treatment choices.

58 A hollow-fibre infection model with two *P. aeruginosa* isolates (MIC 2 and 8 mg/L) with an
59 initial inoculum $\sim 10^8$ colony-forming unit/mL was used to test different amikacin dosing
60 regimens. Three regimens (15, 25 and 50 mg/kg) simulating a blood exposure and a 30 mg/kg
61 regimen simulating the epithelial lining fluid (ELF) for potential respiratory tract infection
62 were tested. Data were described using a semi-mechanistic
63 pharmacokinetic/pharmacodynamic (PK/PD) model. Whole genome sequencing was used to
64 identify mutations associated with resistance emergence.

65 While bacterial density was reduced by >6-logs within the first 12 h in simulated blood
66 exposures, following this initial bacterial kill, there was amplification of a resistant sub-
67 population with ribosomal mutations that were likely mediating amikacin resistance. No
68 appreciable bacterial killing occurred with subsequent doses. There was less (<5-log)
69 bacterial killing in the simulated ELF exposure for either isolate tested. Simulation studies
70 suggest that a dose of 30 and 50 mg/kg may provide maximal bacterial killing for
71 bloodstream and VAP infections respectively.

72 Our results suggest that amikacin efficacy may be improved with the use of high dose therapy
73 to rapidly eliminate susceptible bacteria. Subsequent doses may have reduced efficacy given
74 the rapid amplification of less-susceptible bacterial subpopulations with amikacin
75 monotherapy.

76 Introduction

77 Sepsis or ventilator-associated pneumonia (VAP) caused by *Pseudomonas aeruginosa* is
78 associated with a mortality of between 25 and 50% (1, 2). Furthermore, patients with
79 carbapenem-resistant *P. aeruginosa* infections have an increased risk of death that may be
80 attributed to increasing illness severity, and delayed administration of appropriate antibiotic
81 therapy (3-6). Despite a potential increased mortality with aminoglycoside monotherapy, at
82 least 80% of *P. aeruginosa* isolates remain susceptible to aminoglycosides such as amikacin,
83 therefore they may be prescribed for empiric treatment as part of combination therapy to
84 appropriately extend the spectrum of antibiotic activity in settings with increased resistance
85 rates (3-6).

86 One potential contributing factor to the apparent reduced efficacy of aminoglycosides is
87 suboptimal dosing. Achieving an aminoglycoside maximum concentration ($C_{\max}$) to
88 minimum inhibitory concentration (MIC) ratio ≥ 10 or an area under the-concentration time-
89 curve (AUC) to MIC ratio ≥ 150 reduces mortality and hastens symptom resolution (7, 8)
90 Importantly, the risk of resistance emergence and potential treatment failure may be increased
91 when bacteria are exposed to a $C_{\max}/MIC < 6$ (9). Moreover, in patients infected with
92 carbapenem-resistant, aminoglycoside-susceptible *Klebsiella pneumoniae*, aminoglycosides
93 have been associated with favourable outcomes, particularly when a therapeutic
94 aminoglycoside exposure may be possible at the site of infection (bloodstream, vascular
95 catheters, soft tissues, and urinary tract) (10).

96 Aminoglycoside dose optimization must also consider the potential effect of the bacterial
97 inoculum, the immune response, and the potential toxicity of the dosing regimen.
98 Approximately one-third of patients with VAP have a bacterial burden exceeding 10^8 colony
99 forming units (CFU)/mL (11, 12). Reducing this bacterial burden to $< 1 \times 10^6$ CFU/mL may

100 enable rapid granulocyte mediated bacterial clearance and enhance symptom resolution (11-
101 13). These factors may be particularly important in patients with Gram-negative bacillary
102 pneumonia for two reasons. First, amikacin penetration into the epithelial lining fluid (ELF),
103 the site of infection, is only approximately 10% of the plasma $C_{\max}$ (14). Second, there may
104 be limited treatment options available for multi-drug resistant bacteria should aminoglycoside
105 therapy fail.

106 The aims of this study were twofold. First, to describe and quantify the time course of
107 bacterial killing and emergence of resistance of two *P. aeruginosa* clinical isolates using the
108 dynamic *in vitro* hollow-fibre infection model (HFIM) and semi-mechanistic mathematical
109 modelling. Second, to determine amikacin dosing regimens that may enhance bacterial killing
110 in both the bloodstream and ELF.

111

112 **Materials and Methods**

113 *Antimicrobial agents*

114 Amikacin analytical reference standards (Sigma-Aldrich, batch number LRAA5755) were
115 used for *in vitro* MIC susceptibility testing and preparing amikacin-containing cation adjusted
116 Mueller-Hinton (Ca-MH) agar plates. Commercially available amikacin vials (DBL
117 Amikacin sulphate 500mg/2mL, batch number CO61221AA) stored at 4°C were used for
118 HFIM dosing. Amikacin stock solutions were aseptically prepared in a Class II biosafety
119 cabinet by diluting amikacin with sterile distilled water and storing at -80°C.

120

121

122

123 *Bacterial isolates*

124 Two clinical *P. aeruginosa* isolates (#CTAP40 and #CTAP23) were sourced from critically
125 ill patients. Isolates were stored in Ca-MH broth with 20% glycerol v/v at -80 °C and were
126 grown on Ca-MH agar and incubated at 37 °C for 24 h prior to *in vitro* susceptibility testing
127 and HFIM studies. A 0.5 McFarland bacterial suspension was prepared in sterile water using
128 morphologically similar colonies and diluted in Ca-MH broth to the desired inoculum. For
129 HFIM studies, bacteria were suspended in 40 mL of Ca-MH broth and incubated at 37 °C
130 with constant agitation for a duration of time based on previous growth curves to achieve a
131 final inoculum of approximately 10^8 CFU/mL.

132

133 *In vitro* susceptibility testing

134 Broth microdilution was performed in accordance with Clinical & Laboratory Standards
135 Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing
136 (EUCAST) guidelines (15, 16). Briefly, a volume of bacteria suspended in Ca-MH broth
137 (final inoculum 5.5×10^5 CFU/mL) was added to a 96-well flat or round bottom plate
138 containing serial twofold dilutions of amikacin in Ca-MH broth. Inoculated 96-well plates
139 were incubated for 16-24 h at 37 °C. Round bottom plates were visually inspected for growth;
140 the lowest amikacin concentration with no apparent growth was defined as the MIC. The
141 MIC for the flat bottom plates was determined using a Multiskan FC Microplate Photometer
142 (Thermo Fisher Scientific, Finland), and defined as the concentration with an optical density
143 <0.1 of the growth control. The modal MIC of four replicates within an individual
144 experiment for each method (CLSI and EUCAST) was selected as the isolate MIC.

145

146

147 *Mutation frequency*

148 A 10 mL culture of a 10^2 CFU/mL inoculum was incubated in Ca-MH broth for 24 h at 37°C.
149 Quantitative culturing methods with diluted and undiluted samples were performed on the
150 resultant bacterial growth using both standard Ca-MH agar and amikacin-containing Ca-MH
151 agar (fourfold baseline MIC). The mutation frequency was taken as the ratio of the bacterial
152 concentration growing on amikacin-containing plates to the initial inoculum after incubating
153 for 48 h at 37°C.

154

155 *Hollow-Fibre Infection Model*

156 The HFIM was assembled as described previously using FiberCell Systems polysulfone
157 cartridges (C2011) in all experiments and conducted over 7 days (17, 18). One HFIM
158 experiment was conducted for each dosing regimen and isolate combination with an initial
159 bacterial concentration of 1×10^8 CFU/mL.

160

161 Unbound amikacin blood exposures were simulated using the pharmacokinetic model derived
162 by Romano et al., assuming an 80 kg patient with sepsis, a creatinine clearance of 100
163 mL/min, and 17% protein binding (19, 20). Amikacin dosing regimens of 15, 25, and 50
164 mg/kg once-daily infused over 30-minutes were tested. High 50 mg/kg doses were also tested
165 given that these doses have been previously used clinically (21). The ELF amikacin
166 concentrations and resultant half-life in the HFIM apparatus were approximated using
167 previous aminoglycoside ELF:serum ratios in conjunction with the established concentration-
168 time curves for the blood amikacin exposure (14, 22, 23). In brief, the estimated unbound
169 plasma concentration of amikacin was multiplied by the average ELF:serum penetration ratio
170 (0.12, 0.3, 0.85, 1.14) identified for other aminoglycosides (gentamicin and tobramycin) at

171 the corresponding time points (0.5, 1, 2, 4 h) (14, 22, 23). The ELF half-life (1.92 h) was
172 derived from a non-compartmental analysis of the resultant concentration-time curve over the
173 course of 24 h, which approximates that identified previously (24, 25). A mucin bound
174 fraction of 50% was assumed, representing a likely worst-case scenario (26). An ELF
175 amikacin exposure following an intravenous dose of 30 mg/kg once-daily administered over
176 30-minutes was simulated.

177

178 Samples were periodically removed from the central compartment outlet at 0.25, 0.5, 0.45, 1,
179 2, 3, 4, 6, 8, 10, 12, 24, 25, 30, 36, 48, 49, 54, 60, 72, 73, 78, 84, 96, 120, 144, 145, and 156 h
180 to determine the amikacin concentration for pharmacokinetic analysis. As the central
181 compartment contents rapidly equilibrate with the hollow-fibre cartridge, the concentrations
182 obtained in the central compartment reflect that in the hollow-fibre cartridge. Bacterial
183 quantification was performed with periodic sampling at 0, 2, 4, 6, 8, 11, 24, 35, 48, 59, 72,
184 96, 120, 144 and up to 168 h from the cartridge extra-capillary space. Samples were washed
185 twice in phosphate-buffered saline to minimise antibiotic carry-over. A 100 μ L aliquot of an
186 appropriately diluted bacterial suspension was manually plated onto Ca-MH agar and
187 amikacin-containing Ca-MH agar (fourfold baseline isolate MIC). The limit of quantification
188 was 2- $\log_{10}$ CFU/mL.

189

190 *Drug assay*

191 Amikacin was measured in Ca-MH broth by a validated Liquid Chromatography Mass
192 Spectrometry method. Briefly, 50 μ L of Ca-MH broth sample (neat or diluted) was combined
193 with 50 μ L of water and 20 μ L of vancomycin (50 mg/L) added as the internal standard.
194 Amikacin was extracted using protein precipitation with 50 μ L of trichloroacetic acid (15%,

195 v/v). Samples were centrifuged at 12,000 g for 5-minutes and an aliquot of the supernatant
196 (0.5 μ L) was injected onto a Nexera2 UHPLC system coupled to an 8030+ triple quadrupole
197 MS detector (Shimadzu, Kyoto, Japan). Chromatographic separation was achieved using a
198 Poroshell 120 HILIC column (Agilent, Santa Clara, USA) and a gradient of formic acid 0.2%
199 v/v and acetonitrile with 0.2% formic acid v/v. Detection of amikacin and the internal
200 standard was performed using an electrospray source in positive mode with optimised
201 multiple reaction monitoring conditions for each analyte. Amikacin was monitored at three
202 fragmentation ions (586.25 $\rightarrow$ 163.10, 586.25 $\rightarrow$ 264.15 and 586.25 $\rightarrow$ 425.15) and
203 vancomycin was monitored at two fragmentation ions (725.60 $\rightarrow$ 144.10 and 746.10 $\rightarrow$
204 144.20).

205 Calibration lines of amikacin were quadratic with $1/\text{concentration}^2$ weighting from 0.2 to 10
206 mg/L with a maximum deviation from the nominal concentration of 2.1%. Mean intra-batch
207 accuracy and precision values were -6.2% and 8.3% at 0.8 and 8 mg/L respectively.

208

209 *Whole Genome Sequencing*

210 Bacterial isolates for whole genome sequencing were subcultured onto amikacin-containing
211 (4x baseline MIC) CaMH agar as the resistant bacterial population profile may be transient
212 without the presence of amikacin. Bacterial DNA was extracted without single colony
213 purification to capture population diversity using the DNeasy UltraClean DNA Extraction in
214 accordance with the manufacturer's directions and quantified using spectrophotometry
215 (NanoDrop; ThermoFisher) and fluorometry (Qubit; ThermoFisher). Paired-end DNA
216 libraries were prepared using the Nextera kit (Illumina; Australia) in accordance with the
217 manufacturer's directions. Sequencing was performed using the Illumina Mini-Seq (150 bp
218 paired ends). Improved draft genome assemblies were constructed for the two progenitor

219 strains, #CTAP23 and #CTAP40, using the Microbial Genome Assembler Pipeline (MGAP
220 v1.1)27, and annotated using Prokka v1.12.28 The comparative genomics pipeline,
221 SPANDx29 v3.2.1, was used to determine genomic variation using the either the #CTAP23
222 or #CTAP40 as the reference genome depending on the lineage analysed. Within species
223 mixtures were analysed using the GATK v4.1.0.030 to identify mutations with less than
224 100% allele frequency using the method outlined in Aziz et al. (31).

225

226 *Mathematical Pharmacokinetic/Pharmacodynamic Modelling*

227 All HFIM data from simulated bloodstream exposures were co-modelled using Pmetrics for
228 R version 1.5.2 considering the results of the whole genome sequencing study (32, 33). The
229 final structural model is described by equations 1 to 5 that describe amikacin
230 pharmacokinetics and bacterial growth of three subpopulations. Model diagnostics including
231 the Akaike-information-criteria, log-likelihood, coefficient of determination (R^2) from the
232 observed vs. expected plots, and visual-predictive-checks were used to evaluate and compare
233 models.

234 Equation 1:

$$\frac{dAmk}{dt} = R(1) - \left(\frac{CL}{Vc} \times Amk \right)$$

235 Equation 2:

$$\begin{aligned} \frac{dCFUs}{dt} = & K_{gmax,s} \times CFUs \times \left(\frac{Qmax \times Sub}{Qs + Sub} \right) - CFUs \times K_{killmax,s} \times \left(\frac{\frac{Amk^{Hs}}{Vc}}{EC50_s^{Hs} + \frac{Amk^{Hs}}{Vc}} \right) \\ & - Kds \times CFUs \end{aligned}$$

236 Equation 3:

$$\begin{aligned} \frac{dCFU_i}{dt} = & K_{gmax,i} \times CFU_i \times \left(\frac{Q_{max} \times Sub}{Q_s + Sub} \right) - CFU_i \times K_{killmax,i} \times \left(\frac{\frac{Amk^{Hi}}{V_c}}{EC50_i^{Hi} + \frac{Amk^{Hi}}{V_c}} \right) \\ & - K_{di} \times CFU_i \end{aligned}$$

237

238 Equation 4:

$$\frac{dCFU_r}{dt} = K_{gmax,r} \times CFU_r \times \left(\frac{Q_{max} \times Sub}{Q_s + Sub} \right) - K_{dr} \times CFU_r$$

239 Equation 5:

$$\frac{dSub}{dt} = - \left(\frac{Q_{max} \times Sub}{Q_s + Sub} \right) \times (CFU_s + CFU_i + CFU_r)$$

240 Equation 1 describes amikacin elimination. Equations 2, 3, and 4 describe the bacterial
241 growth, including the theoretical maximal bacterial density and amikacin-mediated killing of
242 the susceptible, intermediate and resistant bacterial populations respectively. Equation 5
243 describes the consumption of an artificial substrate (Sub) required for sustained bacterial
244 growth.

245 Amk amount of amikacin (mg); R(1) amikacin infusion rate (mg/h); CL amikacin clearance;
246 Vc HFIM circuit volume; CFUs, CFUi, and CFUr representing the bacterial burden for the
247 susceptible, intermediate, and resistant *P. aeruginosa* subpopulations respectively (CFU/mL);
248 Kgmax,s, Kgmax,I, Kgmax,r maximal growth rate constants for the susceptible, intermediate,
249 and resistant *P. aeruginosa* subpopulations respectively (log₁₀ CFU/mL/h); Kkillmax,s,
250 Kkillmax,i are the maximum rate of amikacin-mediated bacterial killing (log₁₀ CFU/mL/h);

251 Kds, Kdi and Kdr intrinsic bacterial death rate constants for the susceptible, intermediate and
252 resistant subpopulations ($\log_{10}$ CFU/mL/h); EC50s and EC50i amikacin concentration
253 producing half-maximal bacterial killing for the susceptible and intermediate subpopulations
254 respectively; Sub amount of a fictitious substance required for bacterial growth; Qmax
255 maximum rate of substance use; Qs 50% of maximal substance use; Hs and Hi slope
256 functions for the susceptible and intermediate subpopulations respectively.

257

258 Monte Carlo dosing simulation studies (n=1000) were performed using Pmetrics. Mean
259 pharmacokinetic parameter estimates, as well as standard deviations of the clearance and
260 volume of distribution, were obtained from the study conducted by Romano et al. (19) and
261 applied to the simulations for the pharmacodynamic model. Mean value pharmacodynamic
262 model parameters were estimated for specific isolates and were used for simulations.
263 Moreover, different creatinine clearance values were used to describe patients with lower,
264 normal and high renal amikacin clearance. The fAUC within the first 24 h was calculated
265 employing Pmetrics, which included both the period of infusion and the monoexponential
266 decay. Classification and regression tree analyses (CART) were used to determine the
267 amikacin fAUC (mg.h/L) achieving stasis, 1-log, and 2-log reduction in the bacterial
268 concentration within the first 24 h.

269

270 **Results**

271 *In vitro* susceptibility and mutational frequency studies

272 The modal amikacin MIC for #CTAP23 and #CTAP40 was 2 and 8 mg/L respectively. The
273 mutation frequency for #CTAP23 and #CTAP40 in the presence of 8 and 32 mg/L of
274 amikacin was 6.77×10^{-7} and 1.05×10^{-7} respectively.

275

276 *Hollow-Fibre Infection Model*

277 All intravenous amikacin dosing regimens against a simulated bloodstream *P. aeruginosa*
278 infection resulted in a ≥ 4 -log reduction from the starting inoculum (10^8 CFU/mL) during the
279 first 8 h following the first dose of amikacin (Figure 1). There was no appreciable difference
280 in the rate or extent of bacterial killing between the 15, 25 and 50 mg/kg dosing regimens for
281 #CTAP23 (MIC 2 mg/L) (Figure 1, A). However, there was an approximate 1.5-log
282 difference in the bacterial nadir between the 25 mg/kg and 50 mg/kg dosing regimens against
283 #CTAP 40 (MIC 8 mg/L) (Figure 1, B). The total bacterial burden surpassed the baseline
284 inoculum by 24 h for both isolates following administration of the 15 and 25 mg/kg dosing
285 regimens. Only the 50 mg/kg dosing regimen for both isolates delayed the rate of bacterial
286 regrowth, exceeding the baseline inoculum by 48 h (Figure 1). Bacterial regrowth in the total
287 population was mirrored by bacterial growth on amikacin-containing CaMH agar (Figure 2).
288 The MIC of the bacteria growing on amikacin-containing CaMH agar increased by a
289 minimum of eightfold after seven days of amikacin administration for both isolates tested
290 (Table 1).

291

292 A similar pattern was observed against the simulated ELF exposure. The total bacterial
293 population was reduced by approximately 5-logs, 8 h after the initiation of the amikacin
294 against #CTAP23 (MIC 2 mg/L), which was followed by rapid bacterial regrowth exceeding
295 the baseline inoculum by 24 h, mirrored by growth on amikacin-containing CAMH agar

296 (Figure 1, A; Figure 2). Conversely, there was little appreciable bacterial killing against
297 #CTAP40 (MIC 8 mg/L), yet there was an increase in the growth on amikacin-containing
298 CaMH agar (Figure 2). There was no appreciable bacterial killing following subsequent
299 dosing events after day 1 of amikacin in either the blood or ELF exposures in the HFIM. The
300 observed amikacin concentrations for the simulated unbound plasma and ELF approximated
301 the expected concentrations (Graph A in Figures 3 and 4).

302

303 *Comparative genomic analysis*

304 There were no resistance genes or single nucleotide polymorphisms (SNPs) associated with
305 amikacin resistance identified in the progenitor strains, #CTAP23 or #CTAP40, prior to
306 amikacin commencement. *De novo* SNPs within the *fusA* (*FusA*_{Leu464Val}) and *rplB*
307 (*RplB*_{Gly138Leu}) genes were identified in isolates that were exposed to the 25 and 50 mg/kg
308 daily dosing regimens respectively for #CTAP23 (Table 2). No SNPs were identified
309 following exposure to amikacin 15 mg/kg. SNPs were identified in the *algA* and *tufI*
310 (*TufI*_{Val21Leu}) genes for #CTAP40 following exposure to amikacin; with a small baseline
311 bacterial subpopulation containing an *algA* (*AlgA*_{Ala279Asp}) SNP.

312

313 *Pharmacokinetic/Pharmacodynamic Modelling*

314 Pharmacodynamic parameter estimates are detailed in Table 3. The average total bacterial
315 population Bayesian posterior (model fitted estimate for each individual experimental arm)
316 correlation coefficient (R^2) was 0.97 and 0.78 for #CTAP23 (Figure 3) and #CTAP40 (Figure
317 4) simulated blood exposures respectively. Similar results were found for the resistant
318 bacterial population (average Bayesian posterior R^2 0.97 and 0.95 for #CTAP23 and
319 #CTAP40 respectively).

CART analysis identified similar $fAUC$ and fC_{max} thresholds for bacterial stasis for both isolates over 24 h; correlating with a difference in the $fAUC/MIC$ and the fC_{max}/MIC ratio relative to the isolate MIC (Table 4). However, no threshold was associated with a bacterial kill in the bloodstream of 1- or 2-logs over 24 h for #CTAP23. Amikacin simulated $fAUC$ and fC_{max} ELF exposures were increased relative to plasma for the same bacterial kill over 24 h and was increased for #CTAP23 (MIC 2 mg/L) compared with #CTAP40 (MIC 8 mg/L). The probability of achieving bacterial stasis, 1- and 2-log kill after 24 h is generally high in the ELF and the bloodstream when doses ≥ 30 mg/kg are used (Table 5).

Discussion

This study investigated the bacterial killing and emergence of resistance of two susceptible *P. aeruginosa* isolates exposed to the expected pharmacokinetics of amikacin in blood and ELF. Following an initial bacterial kill of ≥ 4 -logs within the first 8 h, there was extensive bacterial regrowth for both isolates, with negligible bacterial killing following the first dose. Our results support the current EUCAST recommendation that aminoglycosides may be considered for short-term use in combination with another agent until the antibiotic susceptibilities are confirmed and that aminoglycoside dose optimization may enhance bacterial killing and enhance clinical outcomes (34).

In the current study, achieving a blood and ELF amikacin $fAUC$ exposure of approximately >175 ($fAUC/MIC > 21.87$) and >366 mg.h/L ($fAUC/MIC > 45.8$) respectively, may be sufficient to reduce the bacterial burden of some *P. aeruginosa* isolates to $<10^6$ CFU/mL over 24 h. Such an exposure correlates to an amikacin dose of ≥ 30 mg/kg or ≥ 50 mg/kg daily for bloodstream or VAP infections with susceptible *P. aeruginosa* pathogens in patients with

344 normal creatinine clearance (~100 mL/min). However, this threshold may also vary between
345 bacterial isolates as the total bacterial burden within the first 12 h appears to be, in part,
346 mediated by reducing the burden of the susceptible and intermediate-susceptibility bacterial
347 population by achieving the appropriate $fAUC/MIC$ and/or fC_{max}/MIC . Thereafter, a resistant
348 bacterial population for which amikacin has no effect against may emerge. The emergence of
349 resistance is likely dependent on the relative density of the intermediate/resistant
350 subpopulation(s) in the initial total bacterial inoculum (the mutation frequency) and the
351 propensity for mutations to occur that mediate resistance emergence (35). At the high
352 inoculum used in our study, it was expected based on the mutation frequency that a resistant
353 subpopulation existed, which was subsequently amplified following amikacin administration.
354 This may explain the differences in the identified thresholds for a 1- or 2-log reduction
355 between the susceptible isolates used in this study given that the relative
356 susceptible/intermediate/resistant bacterial populations may differ between isolates. Our
357 results are similar to that previously described against *P. aeruginosa* where a simulated
358 gentamicin plasma $C_{max}/MIC \geq 36$ was unable to suppress bacterial regrowth *in vitro* (9).
359 However, against *Acinetobacter baumannii*, an amikacin C_{max}/MIC of 20 suppressed
360 bacterial regrowth, highlighting the variability in response to aminoglycoside exposure that
361 may be, in part, determined by the inoculum size and pre-existing intermediate/resistant
362 subpopulations.

363

364 Higher ELF amikacin $fAUC$ and fC_{max} exposures were required to achieve stasis, 1- and 2-
365 logs of bacterial killing over 24 h, which may be related to the delayed and lower fC_{max}
366 achieved in the ELF relative to the plasma amikacin concentrations following intravenous
367 administration given the expected pharmacokinetic hysteresis between the bloodstream and
368 ELF. Moreover, there was little bacterial killing against #CTAP40 (MIC 8 mg/L) following a

369 simulated intravenous 30 mg/kg dose (Figure 2), suggesting that amikacin monotherapy will
370 have little efficacy against higher MIC isolates.

371

372 The identified PK/PD targets identified in our study differ to those observed in clinical
373 studies. A previous clinical study in critically ill patients receiving intravenous amikacin
374 demonstrated an increased chance of microbial eradication and clinical cure in patients who
375 achieved a $C_{\max}/MIC > 10$ (9). A separate study identified a $fAUC/MIC \geq 150$ mg.h/L
376 correlated with faster symptom resolution in patients with nosocomial pneumonia (7, 8). The
377 identified PK/PD ratios from our simulations in this study and clinical studies may be
378 challenging to achieve with doses < 30 mg/kg (36, 37). As such, high dose amikacin therapy
379 (> 30 mg/kg) may be considered. Limited clinical data exists for such dosing regimens, but
380 doses ≥ 60 mg/kg have been used as part of salvage therapy in conjunction with renal
381 replacement therapy to minimise the probability of toxicity in a small case series (38).
382 Furthermore, the use of a single dose of amikacin in patients with severe sepsis or septic
383 shock may mitigate the risk of nephrotoxicity, which is unlikely to occur for an
384 aminoglycoside duration < 3 days (39). Nevertheless, the use of such high doses would place
385 the patient within an amikacin $fAUC$ exposure that has previously been associated with a
386 significant probability of developing nephrotoxicity; however, this is confounded by the
387 different aminoglycosides used and a prolonged treatment duration (40). This approach
388 should be evaluated in a clinical trial to ensure that both the target PK/PD exposures are met
389 and to assess the potential clinical utility of high dose, short duration therapy in terms of
390 patient morbidity and mortality.

391

392 Despite the achievement of these targets, resistance may still emerge with amikacin
393 monotherapy. Amikacin resistance was identified for both isolates receiving doses up to 50
394 mg/kg within 48 h of amikacin initiation. Mutations affecting the ribosomal binding unit
395 (RplB_{Gly138Leu}), elongation factors (FusA_{Leu464Val}, TufI_{Val211Leu}) and mucoidal phenotype
396 (AlgA_{Ala279Asp}) appear to mediate this resistance, which is consistent with a previous study
397 with tobramycin with similar SNPs within the *rplB* and *fusA* genes that likely inhibit
398 aminoglycoside binding to the 30S ribosomal subunit (41). The relevance of the AlgA mutant
399 is not currently known; however, alteration of alginate production may modify biofilm
400 formation, a known potentiator of antibiotic resistance emergence (42). These mutations were
401 associated with an increased MIC; however, the relative MIC increase was similar following
402 each dosing regimen. Furthermore, a specific mutation was not often consistently identified
403 for all resistant bacterial populations following a specific amikacin dosing regimen. This
404 would suggest that there are either multiple smaller subpopulations that exist, or that
405 alternative resistance mechanisms, such as amikacin efflux exist (43). Nonetheless, given the
406 likely *de novo* emergence of resistance, it is unlikely that subsequent amikacin doses will
407 achieve appreciable further bacterial killing (43). These results would support that notion that
408 amikacin may enhance initial bacterial killing but should be combined with a second agent
409 either empirically or as directed therapy to ensure bacterial eradication and minimise the
410 probability of treatment failure.

411

412 Our study is not without limitations. First, the lack of a simulated immune response *in vitro*
413 limits the external validity when applying our results to clinical practice. Nonetheless, as
414 previously discussed, optimising bacterial killing *in vitro* may generalise to optimal clinical
415 outcomes (44). Moreover, our *in vitro* model and subsequent dosing simulations may best
416 represent an immunocompromised patient. Second, only two clinical *P. aeruginosa* isolates

417 were tested, therefore our results may not generalize to other infecting isolates. Third, the
418 amikacin ELF concentration-time curve is estimated from other aminoglycosides, which may
419 not reflect the exposures achieved for amikacin. This approach may be reasonable given the
420 lack of amikacin-specific data and similar chemical structures between aminoglycosides.
421 Nonetheless, further research detailing the ELF pharmacokinetics of amikacin over a dosing
422 interval are required, thus our results should be considered as hypothesis generating. Fifth, we
423 did not perform WGS on the various phenotypically distinct colonies. This may mean that
424 specific resistance mechanisms may not be appropriately identified if they are present in a
425 sparsely dense bacterial subpopulation. Last, we did not simulate the ELF milieu, which is
426 known to contain mucin, an acidic pH and mucin; factors that are known to impact
427 aminoglycoside-mediated bacterial killing (26, 45-47). The impact of mucin was considered
428 by simulating the estimated unbound amikacin fraction.

429

430 Future amikacin intravenous administration may be with the use of a single high dose (≥ 30
431 mg/kg) of the antibiotic for patients with either bloodstream infections or VAP from multi-
432 resistant pathogens, such as *P. aeruginosa*, to improve the probability of bacterial eradication.
433 However, this must be balanced with ongoing review of the amikacin doses required for
434 clinical effectiveness against *P. aeruginosa* where doses may result in unacceptable toxicity
435 and combinations with other active anti-pseudomonal agents are preferred. Given the likely
436 low efficacy of bacterial killing in the ELF following intravenous administration, alternate
437 amikacin administration routes, such as nebulized therapy, may be considered. Clinical trials
438 are required to define the optimal dosing regimen of amikacin for difficult to treat infections,
439 such as VAP.

440

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645 Table 1: *Pseudomonas aeruginosa* amikacin minimum inhibitory concentrations from
646 isolates grown on amikacin-containing (4x baseline MIC) cation adjusted Mueller-Hinton
647 agar after the 7-day course

Isolate	Amikacin Dose	MIC (mg/L)
#CTAP23	15 mg/kg	32
	25 mg/kg	32
	50 mg/kg	64
	30 mg/kg (ELF)	16
#CTAP40	15 mg/kg	64
	25 mg/kg	128
	50 mg/kg	128
	30 mg/kg (ELF)	64

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670 Table 2: Variation identified in comparison to the initial starting strain in the #CTAP23 and
671 #CTAP40 lineages. Percentages reflect the prevalence of the mutation within the population.

	<i>fusA_2</i> 1390	<i>rplB</i> 413	<i>rplB</i> 412	<i>algA_1</i> 836	<i>tuf1_1</i>
#CTAP23 Baseline	0%	0%	0%		
#CTAP23 15 mg/kg	0%	2%	2%		
#CTAP23 25 mg/kg	57%	4%	4%		
#CTAP23 50 mg/kg	0%	100%	100%		
#CTAP40 Baseline				13%	0%
#CTAP40 15 mg/kg				69%	55%
#CTAP40 25 mg/kg				98%	19%
#CTAP40 50 mg/kg				60%	0%

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690 Table 3: Pharmacodynamic model parameter estimates

Parameter	Abbreviation	#CTAP23	#CTAP40
		Mean (SD)	Mean (SD)
Susceptible Growth Rate constant (log10 CFU/mL/h)	Kgs	1.31 (0.11)	1.08 (0.20)
Intermediate Growth Rate constant (log10 CFU/mL/h)	Kgr	0.40 (0.13)	0.60 (0.26)
Resistant Growth Rate constant (log10 CFU/mL/h)	Kgrr	0.69 (0.11)	0.55 (0.13)
Central compartment HFIM volume (L)	Vc	0.32 (0.01)	0.26 (0.05)
Amikacin clearance (L/h)	Cl	0.03 (0.00)	0.03 (0.00)
Susceptible Killing Rate Constant (log10 CFU/mL/h)	Emaxs	5.34 (1.50)	4.00 (3.01)
Intermediate Killing Rate Constant (log10 CFU/mL/h)	Emaxr	9.43 (3.19)	11.20 (2.10)
Amikacin concentration causing 50% Emaxs (mg/L)	EC50s	11.61 (3.49)	11.10 (2.53)
Amikacin concentration causing 50% Emaxr (mg/L)	EC50r	244.09 (149.73)	349.63 (79.19)
Susceptible Hill Coefficient	Hs	6.00 (4.27)	11.04 (5.80)
Resistant Hill Coefficient	Hr	3.42 (2.47)	7.71 (2.61)
Intermediate population initial condition (CFU/mL)	ICRe	211.05 (119.12)	320.48 (50.57)
Resistant population initial condition (CFU/mL)	ICRRe	29.46 (48.23)	25.81 (16.70)
Maximum substrate consumption	Qmax	0.81 (0.18)	0.59 (0.29)
Maximum available substrate	Substrate	3.33x1010	4.92x1010
		(2.22x1010)	(2.75x1010)
Substrate concentration causing 50% Qmax	Qs	8.15x105	5.3x105 (1.69x105)
		(9.61x104)	
Death rate constant susceptible population	Kds	0.25 (0.15)	0.05 (0.04)
Death rate constant intermediate population	Kdi	0.24 (0.18)	0.02 (0.03)
Death rate constant resistant population	Kdr	0.03 (0.02)	0.11 (0.32)

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692 The mean and standard deviation (SD) for each parameter and isolate were determined using
 693 the average and bootstrapped estimates respectively of the posterior model estimates for each
 694 dosing regimen.

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Table 4: Pharmacokinetic/Pharmacodynamic exposures required for bacterial stasis, 1-log, and 2-log reduction in the total bacterial burden over 24 h.

Isolate	Infection Site	Exposure Target	Stasis	1-log kill	2-log kill
#CTAP40	Blood	$fAUC$	108.81	124.70	174.95
		$fAUC/MIC$	13.60	15.59	21.87
		fC_{max}	24.73	25.86	27.15
		fC_{max}/MIC	3.09	3.23	3.39
	ELF	$fAUC$	328.21	342.69	366.42
		$fAUC/MIC$	41.03	42.84	45.80
		fC_{max}	42.41	47.47	54.17
		fC_{max}/MIC	5.30	5.93	6.77
#CTAP23	Blood	$fAUC$	117.54	-	-
		$fAUC/MIC$	58.77	-	-
		fC_{max}	26.41	-	-
		fC_{max}/MIC	13.21	-	-
	ELF	$fAUC$	342.92	688.54	688.82
		$fAUC/MIC$	171.46	344.27	344.11
		fC_{max}	47.04	42.40	47.81
		fC_{max}/MIC	23.52	21.20	23.91

734 Table 5: Probability of achieving either bacterial stasis, a 1-log reduction, or 2-log reduction
735 in the total bacterial population within 24 h of commencing intravenous amikacin

Isolate	Infection Site	Dose	Renal Function (mL/min)	Stasis	1-log kill	2-log kill
#CTAP40	Blood	15 mg/kg	60	1	1	1
		30 mg/kg	60	1	1	1
		50 mg/kg	60	1	1	1
		15 mg/kg	100	1	0.99	0.89
		30 mg/kg	100	1	1	1
		50 mg/kg	100	1	1	1
		15 mg/kg	140	0.90	0.52	0.16
		30 mg/kg	140	1	1	1
		50 mg/kg	140	1	1	1
	ELF	15 mg/kg	60	0	0	0
		30 mg/kg	60	0.93	0.44	0.03
		50 mg/kg	60	1	1	1
		15 mg/kg	100	0	0	0
		30 mg/kg	100	0.41	0.03	0
		50 mg/kg	100	1	1	1
		15 mg/kg	140	0	0	0
		30 mg/kg	140	0.02	0	0
		50 mg/kg	140	1	1	1
#CTAP23	Blood	15 mg/kg	60	1	0	0
		30 mg/kg	60	1	0	0
		50 mg/kg	60	1	0	0
		15 mg/kg	100	0.99	0	0
		30 mg/kg	100	1	0	0
		50 mg/kg	100	1	0	0
		15 mg/kg	140	0.69	0	0
		30 mg/kg	140	1	0	0
		50 mg/kg	140	1	0	0
	ELF	15 mg/kg	60	0	0	0
		30 mg/kg	60	0.57	0	0
		50 mg/kg	60	1	0	0
		15 mg/kg	100	0	0	0
		30 mg/kg	100	0.06	0	0
		50 mg/kg	100	1	0	0
		15 mg/kg	140	0	0	0
		30 mg/kg	140	0	0	0
		50 mg/kg	140	1	0	0

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740 Figure 1: Total bacterial population for different amikacin dosing regimen in either blood or
741 the epithelial lining fluid (ELF) over 168 h. A; #CTAP23 and B; #CTAP40.

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749 Figure 2: Total bacterial population (filled lines) and resistant population (dashed lines) for
750 #CTAP23 and #CTAP40 in blood (amikacin dosing regimens 15, 25 and 50 mg/kg) and
751 epithelial lining fluid (amikacin dosing regimen 30 mg/kg).

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754 Figure 3: Pharmacokinetic/Pharmacodynamic model observed-predicted fit for isolate
755 #CTAP23. A; amikacin pharmacokinetic data. B and C; Total bacterial population observed

756 vs. predicted values for the population and posterior estimates respectively. D and E;
757 Resistant bacterial population observed vs. predicted values for the population and posterior
758 estimates respectively. Circles #CTAP 23 15 mg/kg; triangles #CTAP23 25 mg/kg; squares
759 #CTAP23 50 mg/kg; hexagons #CTAP23 ELF Exposure; diamonds #CTAP23 Control

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763 Figure 4: Pharmacokinetic/Pharmacodynamic model observed-predicted fit for isolate
764 #CTAP40. A; amikacin pharmacokinetic data. B and C; Total bacterial population observed

765 vs. predicted values for the population and posterior estimates respectively. D and E;
766 Resistant bacterial population observed vs. predicated values for the population and posterior
767 estimates respectively. Circles #CTAP 23 15 mg/kg; triangles #CTAP23 25 mg/kg; squares
768 #CTAP23 50 mg/kg; hexagons #CTAP23 ELF Exposure; diamonds #CTAP23 Control

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