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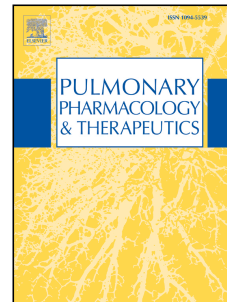

The small quinolone derived compound HT61 enhances the effect of tobramycin against *Pseudomonas aeruginosa in vitro* and *in vivo*

R.T. Amison, M.-E. Faure, B.G. O'Shaughnessy, K.D. Bruce, Y. Hu, A. Coates, C.P. Page

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1 **The small quinolone derived compound HT61 enhances the effect of**
2 **tobramycin against *Pseudomonas aeruginosa in vitro* and *in vivo***
3
4 Amison RT*[1], Faure M-E*[2], O'Shaughnessy BG*[1], Bruce KD[2], Hu Y[3], Coates A[3], Page CP[1]
5 [1] Sackler Institute of Pulmonary Pharmacology, School of Cancer and Pharmaceutical
6 Sciences, King's College London, London, SE1 9NH

7 [2] School of Cancer and Pharmaceutical Sciences, King's College London, London, SE1 9NH

8 [3] Institute of Infection and Immunity, St George's, University of London, Cranmer Terrace,
9 London SW17 ORE, UK.

10 * These authors contributed equally to this study
11

12 **Author for correspondence and reprint requests:**
13 Dr. Richard Amison
14 Sackler Institute of Pulmonary Pharmacology
15 Institute of Pharmaceutical Science
16 Room 5.72 Franklin Wilkins Building
17 Waterloo Campus
18 King's College London
19 London U.K.
20 Phone: +44 2078484796
21 Fax: +44 2078484788
22 **richard.amison@kcl.ac.uk**
23

24 **Running Title: HT61 enhances the antimicrobial activity of tobramycin**

25

26 **Conflicts of Interest**

27 YH and AC are the coinventors of the antibiotic resistance breaker technology, in particular
28 the combination of the quinoline and tobramycin (patent granted). They were the first to
29 test this combination against highly resistant Pseudomonas spp. They originated the
30 concept and performed the background work upon which this work is based

31 AC, YH and CP declare they have equity in Helperby Therapeutics who are developing HT61.
32 CP is in receipt of a grant from Helperby Therapeutics to support Dr Richard Amison for the
33 conduct of the *in vivo* aspect of this study. There are no other conflicts of interest to declare.

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37 **Abstract**

38 HT61 is a small quinolone-derived compound previously demonstrated to exhibit bactericidal activity
39 against gram-positive bacteria including methicillin-susceptible *Staphylococcus aureus* (MSSA) and
40 methicillin-resistant *Staphylococcus aureus* (MRSA). When combined with the classical antibiotics
41 and antiseptics neomycin, gentamicin, mupirocin and chlorhexidine, HT61 demonstrated synergistic
42 bactericidal activity against both MSSA and MRSA infections *in vitro*. In this study, we investigated
43 the individual antimicrobial activity of HT61 alongside its capability to increase the efficacy of
44 tobramycin against both a tobramycin sensitive laboratory reference strain (PAO1) and tobramycin
45 resistant clinical isolates (RP73, NN2) of the gram-negative bacteria *Pseudomonas aeruginosa (P.*
46 *aeruginosa).* Using broth microdilution methods, the MICs of HT61 against all strains were assessed,
47 as well as the effect of HT61 in combination with tobramycin using both the chequerboard method
48 and bacterial time-kill assays. A murine model of pulmonary infection was also used to evaluate the
49 combination therapy of tobramycin and HT61 *in vivo.* In these studies, we demonstrated significant
50 synergism between HT61 and Tobramycin against the tobramycin resistant *P. aeruginosa* strains
51 RP73 and NN2, whilst an additive/intermediate effect was observed for *P. aeruginosa* strain PA01
52 which was further confirmed using bacterial time kill analysis. In addition, the enhancement of
53 tobramycin by HT61 was also evident in *in vitro* assays of biofilm eradication. Finally, *in vivo* studies
54 revealed analogous effects to those observed *in vitro* with HT61 when administered in combination
55 with tobramycin against each of the three *P. aeruginosa* strains at the highest tested dose (10 mg/
56 kg).

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61 ## 1. Introduction

62 *Pseudomonas aeruginosa* is a common pathogen that has been implicated in both hospital and
63 community-aquired infections. Of these different infections, one of the most clinically challenging is
64 the chronic airway infection by *P. aeruginosa* particuarly in individiuals with cystic fibrosis. During
65 exacerbation of clinical symptoms, the aminoglycoside tobramycin is one of the most commonly
66 used antibiotics used in the treatment of *P.aeruginosa* infections (1–5). Once established as a
67 biofilm, *P. aeruginosa* infections are particularly recalcitrant to eradication by tobramycin and other
68 commerical antibiotics. The complexitiy of this situation is further complicated by the existence of
69 many strains of *P. aeruginosa* which display resistance to one or more antibioics, with tobramycin
70 resistance a common occurence (6,7). The resulting health care challenges that are faced by society
71 in the treatment of infectious diseases have been documented extensively (8,9) and with few new
72 classes of antibiotics on the horizon, new strategies are urgently required that refresh or extend the
73 impact of existing antibiotics. One such approach to achieve this is in the identification of novel
74 compounds with the capability to restore the sensitivity of existing antibiotics when used as
75 combination therapies. Such drugs are termed "enhancer compounds".

76 The small quinolone-derived compound HT61, is one such molecule that has previously
77 demonstrated synergy with the aminoglycoside antibiotics neomycin and gentamicin. In these
78 studies, synergy was observed against the gram-positive bacteria MRSA and MSSA *in vitro* and in an
79 *in vivo* model of murine skin infection (10). Whilst HT61 has been shown to enhance the activity of
80 some antibiotics, it has also demonstrated direct bactericidal activity against gram-positive bacteria
81 (11). This bactericidal activity has been proposed to be a result of the cationic charge of HT61
82 partioning into the negatively charged bilayer of the bacterical cell membrane causing structural
83 changes resulting in a loss of membrane integrity and depolarisation. Ultimatley, this severe

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84 membrane damage induces the expulsion of intracellular components and eventually, cell death
85 (12,13). This direct bactericial activity of HT61 has previosuly been used as a topical agent and
86 continues to advance through clinical trials with the ultimate aim of decolonising *S. aureus* from the
87 nasal cavity (14).

88 Despite this ongoing activity investigating the effects of HT61 on gram-positive bacteria, there have
89 been no published studies that have focused on the activity of HT61, either alone or in combination
90 with antibiotics against any gram-negative bacterial species. Our working hypothesis was that,
91 despite the differences in bacterial cell wall structure between gram-negative and gram-positive
92 bacterial species, HT61 may continue to possess direct bactericidal activty and also enhance the
93 activity of aminoglycoside antibiotics such as tobramycin when used in combination against *P.*
94 *aeruginosa*. In this study, the ability of HT61 to enhance the antibacterial activity of tobramycin was
95 assessed against three different strains (RP73, NN2 and PA01) of *P. aeruginosa;* with each strain
96 differing in their tobramycin susceptibility profile. To this end, we combined conventional *in vitro*
97 assays of bactericidal activity such as the chequerboard assay, bacterial time-kill assays and biofilm
98 eradication assays with a *in vivo* murine model of pulmonary infection.

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100 ## 2. Materials and Methods

101

102 ### 2.1. Bacterial Culture conditions and maintenance

103 All bacterial strains were stored at -80°C using the CryoBead system (TSC Ltd., Heywood, UK) and
104 routinely maintained by using Tryptone Soy Agar (TSA; Oxoid, Ltd. Basingstoke, UK). Three *P.*
105 *aeruginosa* strains generously gifted from Dr Alessandra Bragonzi at the Infection and Cystic Fibrosis
106 Unit, San Raffaele Scientific Institute, Milan, Italy. The fully sequenced tobramycin sensitive
107 laboratory reference strain PA01, a multi-drug resistant non-mucoid strain RP73 isolated 17.5 years
108 post onset of infection in a CF patient (multidrug resistant CF isolate) and NN2 a tobramycin
109 resistant CF isolate collected at the onset of chronic colonization (tobramycin resistant CF isolate)
110 (15,16). For all susceptibility assays, Cation-Adjusted Mueller Hinton (CAMH) was obtained from BD
111 Diagnostics (Wokingham, UK) as recommended previously (17). For all *in vivo* studies, all strains
112 were grown on TSA and cultured in Nutrient broth (Oxoid) at 37°C, 120 rpm.

113

114 ### 2.2. Chemicals used in this study

115 All chemicals were purchased from Sigma Aldrich (Sigma-Aldrich, Dorset, UK) unless stated
116 otherwise. All *in vitro* materials were sterilised by autoclaving at 121°C for 20 minutes. HT61-HCl was
117 generously donated by Professor Sir Anthony Coates, St Georges Hospital, London and Helperby
118 Therapeutics, and first dissolved in DMSO (10 g/L). Stock solutions were prepared by the addition of
119 dH_2O to 2.4 g/L prior to sterilisation as effected by 0.22µm filtration (Thermo Fisher Scientific,
120 Loughborough, UK). HT61 solutions were stored at -20°C. Tobramycin stocks were made in dH_2O to
121 give 10 g/L or 50 g/L working concentrations that were sterilised by using filtration at 0.22 µm.
122 Solutions were stored at -20°C.

123

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124 **2.3. Minimum Inhibitory Concentration (MIC) Assay**

125 MICs were determined according to the protocols described previously (18). Serial twofold dilutions
126 of tobramycin and HT61 were prepared in distilled water in flat well polycarbonate 96 well plates
127 (Greiner Bio-One Ltd, Stonehouse, UK). To each well, double strength CAMH broth was added.
128 Colonies from overnight incubation on CAMH agar were resuspended in sterile saline and *c*. $5x10^5$
129 cfu/mL were inoculated. Plates were sealed with a "breathable membrane" (Greiner Bio-One Ltd)
130 and incubated in a wet chamber at 37°C for 16-20 hours. Bacterial load was measured by optical
131 density at 600 nm by using a Fluostar Omega reader (BMG Labtech, Ortenberg, Germany) and used
132 to plot a concentration response curve.

133 MIC experiments are usually determined based of turbidity of the solution, however HT61-HCL is has
134 been shown precipitate in CAMH broth at high concentrations that resulting in a high absorbance
135 background. Therefore we used the metabolic dye Resazurin to overcome this background
136 precipitation as has been previously reported (19). Briefly, Resazurin sodium salt was added to a final
137 concentration of 0.15 mM and plates were incubated for an additional 2-4 hours. MICs were then
138 defined as the lowest concentration inhibiting bacterial activity using the conversion of resazurin
139 (blue) to resurofin (pink). Experiments were performed three times in duplicate.

140

141 **2.4. Synergy assessment by Checkerboard assay**

142 The chequerboard methods was used for the measurement of potential synergy from combinations
143 of HT61 with tobramycin against each of the 3 strains of *P. aeruginosa* as previously described
144 (19,20). Briefly, the combinations of Tobramycin and HT61 were prepared using 96-well plates using
145 drugs concentrations starting from 2 fold higher than their MIC values and the serially diluting 2-fold
146 to zero in sterile flat well polycarbonate plates (Greiner Bio-One Ltd) to create 64 combinations. The

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147 experiment was then performed using the MIC methodology as described above. The growth/no
148 growth interface was then determined using the visual colour change of resazurin (blue) in to the
149 pink resorufin product and used to plot isobolograms and for the calculation of the fractional
150 inhibitory concentration (FIC) as a measure of the effects of combination therapies.

151 The sum of FICs or FIC indexes (FICIs) were calculated as ΣFICA+B= $FIC_A + FIC_B$ where $FIC_A = MIC_{A+B}$ /
152 MIC_A and $FIC_B=MIC_{A+B} / MIC_B$.

153 The Loewe additivity theory was used with a lower cut off to determine synergy (21). The interaction
154 of the combination was defined as showing synergy if the FICs were ≤0.5, additivity or no interaction
155 if the FIC was >0.5 but <4.0 and antagonism if the FIC was >4.0 (20).

156

157 **2.5. Synergy assessment by Bacterial Time Kill Assays**

158 To investigate any synergistic effects on the rate of killing, time-kill assays were performed with
159 HT61 and tobramycin as previously described (22,23). Bacteria ($5x10^5$ cfu/mL) were cultured in 10
160 mL of CAMHB in glass flasks in the presence of tobramycin and/or HT61 at sub-inhibitory
161 concentrations. Cultures were incubated at 37°C for 24 hours under continuous shaking conditions
162 (130 rpm). Bacterial viability was determined by collecting aliquots at 0, 4, 8, 12 and 24 hours.
163 Samples of bacterial suspensions were then serially diluted in sterile saline and plated on TSA. Plates
164 were subsequently incubated at 37°C for 16-18 hours, and colony forming units counted.

165

166 **2.6. Minimum biofilm concentration eradication assay**

167 Effect of combination therapy was studies on biofilms by using the Calgary devices as previously
168 described with minor modifications (24). One colony was inoculated in CAMH and incubated (37°C,
169 130 rpm). After approximately 20 hours, cells were washed twice by centrifugation (5,000*g*, 4°c) and

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170 resuspension in sterile 0.9% NaCl. Bacterial suspensions were diluted to 10^5 cfu/mL in CAMH and
171 inoculated in a 96 well plate (150 µL per wells). Plate lids were replaced with the Nunc-TSP 96 Pin lid
172 and biofilms were left to form for 18-20 hours under shaking (110 rpm) in a wet atmosphere.
173 Established biofilms on pins were washed with 200 µL of sterile 0.9% NaCl and transferred into a
174 challenge plate. Challenge plates were made of serial dilutions of tobramycin supplemented with
175 HT61 at 25, 50 and 100 µg/mL in CAMH (200 µL per wells). For each plate, controls of tobramycin
176 and HT61 alone were present, as well as sterility controls. Biofilms were challenged for 18-20 hours
177 under the same condition (37°C, 110 rpm, wet atmosphere). Following challenge, biofilms were
178 placed in an inhibitor free environment (CAMH, 200 µL per wells), left to equilibrate at room
179 temperature for 30 minutes and sonicated at high intensity for 30 minutes. Sonication efficiency
180 were visually checked by crystal violet 0.1 % staining. Recovery plates containing detached biofilms
181 were incubated for a further 24 to 48 hours and used to determine MBEC (minimal biofilm
182 eradication concentration), where an MBEC was defined as the lowest concentration to inhibit
183 growth as previously recommended.

184

185 **2.7. Murine Model of Pulmonary Infection**

186 All animal experiments were performed under the authority and approval of the U.K Home Office
187 outlined in the Animals (Scientific Procedures) Act 1986 with local ethical approval from King's
188 College London (project license PPL 70/8279).

189 Bacterial embedded agar beads were prepared using the protocol described previously (25). Briefly,
190 bacterial cultures were prepared by inoculating a TSA plate with a stock cryobead and incubating at
191 37°C for 24 hours under static conditions. 24 hours prior to use, secondary overnight cultures were
192 prepared by inoculating 20ml of TSB with 2-3 bacterial colonies and subsequently incubated at 37°C
193 for 16 hours under rotary conditions of 120rpm. Bacteria were embedded into agar beads by mixing
194 the secondary overnight culture with molten TSA at a 1:9 ratio, which was then spun into mineral oil,

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195 previously heated to 50°C. The preparation was cooled and centrifuged at 2700 g and the remaining
196 mineral oil was removed by washing with sterile PBS. Bacterial content of the beads was then
197 quantified on TSA plates and diluted to achieve $2x10^7$ cfu/mL using sterile PBS, to deliver a final dose
198 of $1x10^6$ cfu/mouse.

199 Animals were housed in filter top cages under standard conditions of 22±2°C with a 12:12 light: dark
200 cycle. All animals were provided with food and water *ad libitum,* and wood shavings, shredded paper
201 and cardboard tubes were provided for environmental enrichment. All animals were provided with a
202 minimum acclimatization period of 7 days upon arrival before the commencement of the study.
203 Male C57/Bl6J mice (8 weeks, Harlan, United Kingdom) were anesthetized with inhaled isoflurane
204 and inoculated with $1x10^6$ cfu/mouse bacterial embedded agar beads via oropharyngeal infection
205 (*o.a*). Sham mice were inoculated with sterile PBS embedded agar beads via the *o.a*. route of
206 administration, as a control. Animals were weighed and monitored daily for signs of pain and
207 distress. Animals that lost more than 20% of their body weight throughout the duration of the study
208 were euthanised. 24 hours post infection, mice were treated with either saline vehicle, HT61,
209 Tobramycin or a combination treatment of HT61 and Tobramycin via intraperitoneal (*i.p.*) injection.
210 48 hours post infection, lungs were aseptically removed and homogenized in 2mL sterile saline.
211 Serial dilutions of lung homogenates were performed, and appropriate dilutions plated onto TSA
212 plates and incubated for 24 hours under static conditions. Colonies were manually quantified and log
213 (CFU/mL) calculated.

214

215 **2.8. Statistical Analysis**

216 Chequerboard analysis of synergy was used to identify a fractional inhibitory concentration (FIC)
217 index, where synergy was determined if the FIC was ≤0.5. Additivity or no interaction was described
218 if the FIC was >0.5 but <4.0 and antagonism if the FIC was >4.0. *In vitro* Time-kill analyses of data

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219 were presented as mean ± standard error of the mean (SEM) and analysed by using GraphPad Prism
220 8.0. Due to experimental variation, MBEC were expressed as mean of 9 to 12 replicates. These data
221 were normally distributed as shown by the QQ plots in **S2 Fig**. They were therefore eligible for
222 parametric testing. Differences between untreated control and each of the treated groups (25, 50 or
223 100 µg/mL) were analysed by using an unpaired student t-test with Welch's correction. The one-way
224 analysis of variance and Sidak's multiple comparisons post-test were used for comparisons of data.
225 All *in vivo* data are presented as mean ± standard error of the mean of log cfu/ml and analysed using
226 GraphPad Prism 8.0. The one-way analysis of variance and Sidak's multiple comparisons post-test
227 were used for comparisons of data. P values of less than 0.05 were determined to demonstrate a
228 significant difference.

229
3. Results

230
3.1. Differences in MIC values for HT61 and tobramycin for strains of *P. aeruginosa*

231 The MICs of HT61 and Tobramycin were determined against 3 different strains of *P. aeruginosa* with
232 different susceptibility profiles to Tobramcyin. MICs values of HT61 ranged between 50 and
233 100µg/ml against each of the 3 strains (PA01: 100 µg/ml; NN2: 100 µg/ml; RP73: 50 µg/ml, **Table 1**).
234 Previous research has reported differential sensitivity of these 3 *P.aeruginosa* strains to Tobramycin,
235 with PA01 reported as a tobramycin sensitive strain, RP73 reported as a multidrug resistant strain
236 and NN2 reported as Tobramycin resistant (15). In our studies, in agreement with the previously
237 reported susceptibility profiles, we also reported marked differences in the MIC values of
238 Tobramycin to each of these strains with a range of 0.4-100µg/ml (PA01: 0.4 µg/ml; RP73: 3 µg/ml;
239 NN2: 100 µg/ml, **Table 1**). HT61 was more effective as demonstrated by the smaller MIC in CAMH in
240 comparison to LB and TSB for all three strains. Similarly, an enhanced susceptibility to tobramycin
241 was also observed in CAMH for strains PAO1 and RP73.

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244 **3.2. Chequerboard analysis revealed HT61 and tobramycin synergy against *P. aeruginosa***
245 **strains RP73 and NN2**

246 We have previously reported that HT61, demonstrated synergism against gram-positive bacteria *in*
247 *vitro* when used in combination with a number of aminoglycoside antibiotics, including neomycin,
248 gentamicin and mupirocin (10). We therefore extended this work by assessing the combined
249 activities of HT61 and Tobramycin against the 3 strains of *P.aeruginosa*. The checkerboard assay was
250 used to assess the effect of combined tobramycin and HT61 treatment on *P. aeruginosa* growth,
251 using 64 combinations of tobramycin and HT61. FICs were determined by using the growth/no-
252 growth interface. This gave the MIC values for each agent when used alone and in combination. A
253 representative image for this can be observed for the multi-drug resistant strain RP73 in **Fig 1A**. In
254 this study, synergy was defined as $\Sigma FICs \leq 0.5$ (20). **Fig 1B** demonstrates the FIC indexes for each
255 strain. All details of MICs and FIC calculations are shown in **Supplementary Table 1**. The impact of
256 HT61 and tobramycin interactions was shown to be indifference/additive for strain PAO1 ($\Sigma FIC =$
257 0.65 ± 0.073), whilst synergistic for strains RP73 ($\Sigma FIC = 0.425 \pm 0.05$) and NN2 ($\Sigma FIC = 0.425 \pm 0.073$).

258

259 **3.3. Time-kill analysis reveals synergy between HT61 and tobramycin against *P.***
260 ***aeruginosa* strains PAO1, RP73 and NN2**

261 The bactericidal activities of HT61 and tobramycin in combination were assessed in bacterial time-kill
262 assays over a 24 hours timescale. **Fig 2** shows the time kill assay data obtained for *P. aeruginosa*
263 strains PAO1 (**A**), RP73 (**B**) and NN2 (**C**). HT61 (black squares) and tobramycin (black circles) were
264 used at subinhibitory concentrations, which did not significantly impact the final number of *P.*
265 *aeruginosa* cells (growth to approximately 10^10 CFU/mL for all strains examined). Assays that

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266 combined both agents at the same concentration are shown as black triangles. Synergy was
267 subsequently defined as when the difference in bacteriasl numbers exceeded two log orders, when
268 comparing a combination of agents to single agents. Strong evidence for synergy was identified after
269 eight hours for strain PAO1 (**Fig 2A**), with no cfu's detected for PA01 after 12 hours following
270 combination treatment of HT61 and Tobramycin in comparison to 10^8 cfu/ml following HT61
271 monotherapy and 10^4 cfu/ml following Tobramycin monotherapy at the same timepoint.

272 For strain RP73 (**Fig 2B**), no difference between HT61 and Tobramycin monotherapies were
273 observed at any timepoint. However, combination therapy of HT61 and Tobramycin demonstrated a
274 2 log fold reduction in recovered bacterial cfu at 8 hours (HT61 Montherapy: $4.65x10^5$ cfu/ml;
275 Tobramycin Monotherapy: $3.93x10^5$ cfu/ml; Combination therapy: $1.2x10^3$ cfu/ml) indicating synergy
276 between the two compounds. The same trend was also observe for the Tobramycin resistant strain
277 NN2 (**Fig 2C**), where no difference between HT61 and Tobramycin monotherapies were observed,
278 whilst combination therapy of HT61 and Tobramycin once again demonstrated a 3 log fold reduction
279 in recovered bacterial cfus at 8 hours (HT61 Montherapy: $3.89x10^5$ cfu/ml; Tobramycin
280 Monotherapy: $8x10^5$ cfu/ml; Combination therapy: $2x10^2$ cfu/ml) from 8 hours onwards.

281

282 **3.4. HT61 significantly enhances tobramycin mediated biofilm eradication**

283 Since *P. aeruginosa* infections are typically associated with bacteria persisting as biofilms, the impact
284 of combinations of HT61 and tobramycin was also investigated on these structured forms of growth.
285 Firstly, we investigated whether biofilm establishment *in vitro* was affected by singular treatment
286 with either HT61 or tobramycin through the checkerboard assay system. In our studies we observed
287 similar FICs values similar to those obtained from produced in the non-biofilm grown samples(data
288 not shown).

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289 Secondly, we aimed to investigate the effect of tobramycin and HT61 on already established
290 biofilms. Biofilms, formed in inhibitor free conditions, were challenged with tobramycin and HT61.
291 The period of recovery required was used to determine the minimum biofilm eradication
292 concentration (MBEC). HT61 given as a monotherapy had no impact on bacterial cell numbers in
293 previously established biofilm structures (data not shown). As such, the impact of different
294 concentrations of tobramycin in the presence of fixed levels of HT61 (25, 50 or 100 µg/mL) was
295 assessed (**Fig 3**). For all strains tested, the addition of HT61 significantly enhanced the ability of
296 tobramycin to eradicate biofilm located cells, as shown by the decreased MBEC. For strain PAO1 (**Fig**
297 **3A**), the lowest concentration of tobramycin required to eradicate biofilms was 43.75 ± 4.49 µg/mL.
298 The addition of 25, 50 or 100 µg/mL of HT61 significantly reduced this MBEC to 26.56 ± 4.49 µg/mL
299 ($p<0.05$), 15.89 ± 2.48 µg/mL and 15.36 ± 2.59 µg/mL respectively ($p<0.0001$). For strain NN2 (**Fig**
300 **3B**), MBEC for tobramycin alone was above 25 g/L. As the solubility limit was reached, tobramycin
301 MBEC was assumed to be 50 g/L for those replicates. The tobramycin MBEC alone was therefore
302 estimated at 43.75 ± 3.26 g/L, which was significantly reduced ($p<0.0001$) by at least 4-fold when
303 HT61 was added (10.94 ± 1.56 g/L, 9.90 ± 1.14 g/L, 6.77 ± 0.85 g/L). The strongest level of
304 enhancement on biofilm eradication was observed with RP73 (**Fig 3C**). When used as a single agent,
305 200 µg/mL tobramycin was required to eradicate RP73 biofilm structures, whilst HT61 at 25, 50 or
306 100 µg/mL was shown to lower the tobramycin MBEC to 53.75 ± 10.87 µg/mL (3.7 fold), 33.13 ± 6.91
307 µg/mL (6-fold) and 25.0 ± 6.04 µg/mL (8-fold) respectively.

308

309 **3.5. HT61 showed no bactericidal activity against any tested bacterial strain *in vivo***

310 The *in vitro* data described in sections 3.1-3.4 described an ability of HT61 to enhance the activity of
311 tobramycin against both RP73 and NN2. We therefore investigated whether this enhancement was
312 replicated in *in vivo* studies using a murine model of pulmonary infection with *P. aeruginosa.* Dose-
313 response titrations of HT61 were performed towards infections with all three strains of *P.*

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314 *aeruginosa* described above, in order to determine whether HT61 monotherapy demonstrated any
315 bactericidal activity *in vivo*. In all studies, inoculation with 1x10^6 cfu RP73/NN2/PA01 per mouse
316 resulted in significant increases in bacterial cell numbers when compared to sham controls (Sham:
317 0.00 ± 0.00 log cfu/ml vs. RP73: 6.16 ± 0.22 log cfu/ml, P < 0.001; NN2: 4.85 ± 0.18 log cfu/ml, P <
318 0.001; PA01: 5.90 ± 0.03 log cfu/ml, P < 0.001, **Figs 4A-C**). Single systemic treatment with 0.1, 1 and
319 5mg/kg HT61 *i.p.* failed to demonstrate any reduction in pulmonary bacterial load when compared
320 to vehicle treated controls at 48 hours against any of the three *P. aeruginosa* strains tested; RP73
321 (Vehicle: 6.16 ± 0.22 log cfu/ml vs 0.1mg/kg: 5.91 ± 0.12 log cfu/ml; 1mg/kg: 6.45 ± 0.12 log cfu/ml;
322 5mg/kg: 6.64 ± 0.41 log cfu/ml), NN2 (Vehicle: 4.85 ± 0.18 log cfu/ml vs 0.1mg/kg: 5.34 ± 0.32 log
323 cfu/ml; 1mg/kg: 5.12 ± 0.43 log cfu/ml; 5mg/kg: 5.80 ± 0.69 log cfu/ml) or PA01 (Vehicle: 5.90 ±
324 00.03 log cfu/ml vs 0.1mg/kg: 6.73 ± 0.20 log cfu/ml; 1mg/kg: 5.74 ± 0.14 log cfu/ml; 5mg/kg: 5.74 ±
325 0.33 log cfu/ml). This suggested that unlike in previous studies performed against gram-positive
326 bacteria, HT61 has no direct anti-bacterial activity against gram-negative *P.aeruginosa.*

327

328 **3.6. HT61 significantly enhanced the efficacy of tobramycin against the *P. aeruginosa***
329 **strains RP73, NN2 and PA01 *in vivo***

330 Our *in vitro* experiments demonstrated clear synergy between HT61 and tobramycin when used as a
331 combination therapy against *P. aeruginosa* strains RP73 and NN2, but not PA01. We therefore
332 subsequently assessed whether this *in vitro* finding could be translated *in vivo* using a murine model
333 of pulmonary infection.

334 Initial dose-response experiments were performed to identify a sub-threshold dose of tobramycin
335 with which failed to reduce bacterial numbers retrieved from the lungs 48 hours post infection when
336 compared to vehicle controls **(Supplementary Figure 1)**. From these experiments, 100mg/kg

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337 systemic tobramycin was selected for the drug resistant *P. aeruginosa* strains RP73 and NN2, whilst
338 50mg/kg was selected for *P. aeruginosa* reference strain PA01.

339 In the first series of the combination studies, mice were concomitantly treated with subthreshold
340 tobramycin doses as previously identified and 1mg/kg HT61. Supporting our earlier *in vitro* and *in*
341 *vivo* experiments described above, treatment with 1mg/kg HT61 alone demonstrated no reduction
342 in bacterial numbers 48 hours post treatment when compared to vehicle for RP73 (Vehicle: 5.54 ±
343 0.01 log cfu/ml vs 1mg/kg HT61: 5.65 ± 0.01 log cfu/ml), NN2 (Vehicle: 6.59 ± 0.21 log cfu/ml vs
344 1mg/kg HT61: 6.54 ± 0.20 log cfu/ml) or PA01 (Vehicle: 4.70 ± 0.13 log cfu/ml vs 1mg/kg HT61: 4.97
345 ± 0.06 log cfu/ml). Furthermore, monotherapy with the subthreshold dose of Tobramycin had no
346 effect on bacterial numbers when compared to vehicle for RP73 (Vehicle: 5.54 ± 0.01 log cfu/ml vs
347 100mg/kg tobramycin: 5.56 ± 0.01 log cfu/ml), NN2 (Vehicle: 6.59 ± 0.21 log cfu/ml vs 100mg/kg
348 tobramycin: 6.24 ± 0.24 log cfu/ml) or PA01 (Vehicle: 4.70 ± 0.13 log cfu/ml vs 50mg/kg Tobramycin:
349 5.06 ± 0.11 log cfu/ml) (**Fig 5A and B**). However, when mice were treated with a combination of both
350 1mg/kg HT61 and tobramycin, a significant reduction in bacterial numbers was observed against the
351 multi-drug resistant strain RP73 (Vehicle: 5.54 ± 0.01 log cfu/ml vs 1mg/kg HT61 + 100mg/kg
352 Tobramycin: 4.51 ± 0.12 log cfu/ml, $P < 0.001$) and the tobramycin resistant strain NN2 (Vehicle: 6.59
353 ± 0.21 log cfu/ml vs 1mg/kg HT61 + 100mg/kg tobramycin: 5.49 ± 0.33 log cfu/ml, $P < 0.05$). In
354 contrast, this finding was not observed for the tobramycin sensitive strain PA01 (Vehicle: 4.70 ± 0.13
355 log cfu/ml vs 1mg/kg HT61 + 50mg/kg tobramycin: 5.00 ± 0.06 log cfu/ml) (**Fig 5C**)

356 Next, we investigated whether the inability of HT61 to potentiate the activity of tobramycin against
357 PA01 was due to an insufficient dose of HT61 used in the combination treatments. We therefore
358 replicated the above studies using an increased 10mg/kg HT61 in the combination treatment with
359 tobramycin. With this increased dose of HT61, we observed significant reductions in pulmonary
360 bacterial numbers against vehicle control mice 48 hours post infection with all strains, RP73 (Vehicle:
361 4.81 ± 0.25 log cfu/ml vs 10mg/kg HT61 + 100mg/kg tobramycin: 2.57 ± 0.36 log cfu/ml, $P < 0.001$),

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362 NN2 (Vehicle: 4.49 ± 0.14 log cfu/ml vs 10mg/kg HT61 + 100mg/kg tobramycin: 3.74 ± 0.31 log
363 cfu/ml, P < 0.05) and PA01 (Vehicle: 4.96 ± 0.11 log cfu/ml vs 10mg/kg HT61 + 50mg/kg tobramycin:
364 3.41 ± 0.46 log cfu/ml, P < 0.05) **(Figs 6A-C).**

365 We were interested as to whether or not the significant reductions observed *in vivo* by combining
366 HT61 with tobramycin was due to an additive effect or due to synergistic interactions between the
367 two drugs. We therefore calculated the expected reductions in bacterial numbers if it were due to
368 an additive effect (**Supplementary Table 2**). Here, the reductions in bacterial numbers observed for
369 all 3 strains suggested the possibility that a greater than additive effect was observed between HT61
370 and tobramycin, which supported our *in vitro* data.

371

372 ## 4. Discussion

373 The properties of compounds such as HT61 have been of growing interest in terms of clinical utility.
374 In this study, we found that HT61 had little or no discernable antibacterial activity against the gram-
375 negative pathogen *P. aeruginosa* in contrast to the previously described activity observed against
376 gram-positive bacteria (11). However, the key findings of this study were the identification of
377 significant synergistic activities between HT61 and the classical aminoglycoside antibiotic tobramycin
378 against *P. aeruginosa.* These findings have a particular bearing in terms of the clinical management
379 of airway infection by *P. aeruginosa* for individuals with cystic fibrosis. Whilst novel therapies are
380 emerging that address the fundamental genetic defect associated with cystic fibrosis, it is equally
381 important to improve existing antibiotic approaches towards the clearance of chronic infections by
382 this pathogen, particularly in patients colonized with strains resistant to treatment with antibiotics.

383 In this study, 3 different strains of *P. aeruginosa* were considered including the multi-drug resistant
384 (RP73) and tobramycin resistant (NN2) strains (15) alongside PAO1 as a reference laboratory strain.

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385 As such, these strains represented a range of known phenotypic responses to tobramycin. The
386 observation that HT61 in combination with tobramycin produced a synergistic effect *in vitro* against
387 the strains considered as resistant to tobramycin was therefore encouraging. In turn, this provided a
388 strong rationale for the assessment of the antibacterial activity of combinations of HT61 and
389 tobramycin *in vivo* using a murine model of pulmonary infection. As such, our observations have
390 extended previous work with HT61 where this small quinolone derived compound has demonstrated
391 activity against gram-positive species, including *Staphylococcus aureus* (both MSSA and MRSA) *in*
392 *vitro* and in a wound infection model (10,11,26,27). Importantly, in these studies HT61 was also
393 observed to enhance the bactericidal activity of existing antibiotics including gentamicin and
394 neomycin against both MSSA and MRSA in addition to its own individual bactericidal capabilities
395 (11,28). Extending from these studies our focus here was on *P. aeruginosa*, which demonstrates
396 significant resistance to current therapy. The finding in this study that both a tobramycin resistant
397 and multi-drug resistant strain of this species were rendered sensitive – as if "restored" – was
398 therefore a novel and exciting finding and the potential clinical benefits of such a finding are clear.
399 Through the identification of the requirement of HT61 in this combinational role, we propose this
400 class of antibacterial agent as an enhancer of the efficacy and impact of conventional antibiotics, and
401 therefore propose the definition of *'antibiotic enhancer'* as a substance that in relatively low
402 concentration extends the antibacterial activity of a conventional antibiotic, despite having no or
403 low-level antibacterial activity in itself.

404 Given the concern over the lack of novel antibiotics in the pipeline, alternate strategies designed to
405 maximise and enhance the activity and in turn longevity of existing antibiotics are important.
406 Borrowing the same terminology as for antibiotic susceptibility, enhancers could either be narrow or
407 broad in their spectrum. The ability of HT61 therefore to render RP73, a nearly tobramycin resistant
408 strain (MIC of 3µg/mL), susceptible to tobramycin therapy (MIC reduced to 0.6µg/mL), both *in vitro*
409 and *in vivo* is therefore highly important. Such enhancer strategies would avoid scenarios whereby
410 resistance to an antibiotic is overcome by simply increasing the dose of the antibiotic prescribed.

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411 This is of particular relevance for aminoglycosides such as tobramycin, as it is neither feasible or
412 ethical to significantly increase the doses used due to their systemic toxicity profiles, as in addition
413 to their ototoxic and nephrotoxic side effects, chronic kidney disease has also been associated
414 chronic tobramycin treatment, in individuals with cystic fibrosis (29–32). As an alternative to simply
415 increasing antibiotic plasma levels, the use of multiple antibiotics in combination has been proposed
416 as a strategy capable of slowing the emergence of antimicrobial resistance whilst also shortening the
417 required duration of therapy (33). Antibiotics from a number of different classes have previously
418 been reported to show synergistic effects against tobramycin resistant strains of *P. aeruginosa* (34),
419 therefore enhancer strategies (or antibiotic resistance breakers), featuring low or no direct antibiotic
420 impact, may therefore be less susceptible as a target for resistance emergence and could be a viable
421 alternative approach (35,36)

422 Resistance, emerging or intrinsic, is a feature of the pathogen *P. aeruginosa* (37). Despite the layered
423 nature of the resistance characteristic of *P. aeruginosa*, compounds able to compromise the integrity
424 of bacterial cell membranes are likely to enhance antibiotic penetration. The mechanism by which
425 HT61 may enhance the activity of classical antibiotics through its non-specific targeting the anionic
426 lipids in the bacterial membrane due to the negative charge of HT61. In the case of *S. aureus* such
427 anionic lipids may include phosphatidylglycerol (PG) and the zwitterionic phosphatidylethanolamine
428 (12,13). In targeting these lipids, HT61 induces rapid partitioning of the lipid bilayer into a monolayer
429 causing structural changes thus impairing membrane integrity leading to depolarization and
430 catastrophic membrane damage (12,13). In contrast to previous studies, where HT61 has shown
431 bactericidal activity against strains of *S. aureus* (10,11), HT61 treatment as a monotherapy had little
432 or no bactericidal effects on any of the tested strains of *P. aeruginosa* in any of our *in vitro* or *in vivo*
433 models. This may be due to differences in the membrane lipid composition between the two species
434 (38,39) with cationic membrane acting agents such as HT61 showing greater activity against *S.*
435 *aureus* (40).

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436 Using a pharmacodynamic model, Bulitta *et al.* accounted for two killing mechanisms associated
437 with aminoglycoside use against *P. aeruginosa* (41). Delayed killing was attributed to the effect of
438 tobramycin on bacterial protein synthesis whilst immediate killing was attributed to disruption of
439 the outer membrane (41). It is possible that our observation of increased tobramycin activity when
440 combined with HT61 is as a result of the initial disruption of the outer membrane by tobramycin
441 increasing the exposure of the anionic phospholipids on the inner cytoplasmic membrane to HT61.
442 The resulting disruption of both membranes would increase the permeability of the gram-negative
443 bacterial membranes to tobramycin, resulting in an elevated intracellular tobramycin concentration
444 enhancing the bactericidal activity through its effects on protein synthesis (10,11). Whilst the
445 mechanism of action of HT61 remains open for discussion and further studies are required to fully
446 understand the mechanism of action of HT61 in the potentiation of tobramycin against gram-
447 negative bacteria, it is also important to consider the impact on the mode of growth of *P.*
448 *aeruginosa*. Here, the impact on biofilm formation, as well as the impact of HT61/tobramycin on
449 existing biofilms was also examined. Whilst HT61 had no impact on either establishing or established
450 biofilm structures in our tested concentration range, when treated in combination with tobramycin
451 we demonstrated a 10-fold reduction in the MBEC observed with tobramycin alone.

452 These studies therefore demonstrate an exciting enhancement of tobramycin's activity; however,
453 we acknowledge that our studies used an extended range of assays, the findings are limited by the
454 assessment of a single antibiotic. Whilst we used three individual strains of *P. aeruginosa* covering a
455 range in susceptibility to a tobramycin the assessment of HT61's ability to enhance the bactericidal
456 activity of additional antibiotics would further strengthen the findings detailed in this study.

457 In conclusion, the combination of tobramycin with HT61 demonstrated significant potentiation of
458 bactericidal activity when compared to tobramycin as a monotherapy against both tobramycin
459 sensitive and resistant strains of *P. aeruginosa,* in both *in vitro* assays and a *in vivo* model of lung
460 infection. This indicates the potential benefits of combination treatments using enhancer

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461 compounds, such as HT61 alongside conventional antibiotics including tobramycin in the treatment
462 of antibiotic resistant gram-negative infections. Such enhancer strategies would support the use of
463 lower doses of the aminoglycoside antibiotics significantly reducing their associated toxicity profiles
464 thus providing a potentially novel way of targeting the ongoing global issue of antimicrobial
465 resistance.

466

467

468

469 Author Contributions

470 RA, MF, BGO, KB, YH, AC and CP were involved with the conception, hypotheses delineation, and
471 design of the study. RA, BGO and CP were involved with acquisition of the *in vivo* data, analysis and
472 interpretation of the data. MF and KB were involved with acquisition of the *in vitro* data, analysis
473 and interpretation of the data. RA wrote the manuscript. All authors were involved with manuscript
474 revision prior to submission.

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476 6. References

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***P. aeruginosa* strain**	**Antibiotic resistance**	**MIC (µg/mL)**	
		Tobramycin	**HT61**
PAO1	**Tobramycin sensitive (*15*)**	**0.4**	**100**
RP73	**Multi resistant (*15*)**	**3**	**50**
NN2	**Tobramycin resistant (*15*)**	**100**	**100**

631
632 **Table 1: *P.aeruginosa* susceptibility profile to tobramycin and HT61**
633 MIC values derived from dose-response curve data for HT61 and tobramycin with three
634 strains of *P. aeruginosa* PA01, RP73 and NN2. Here MIC values are displayed as µg/ml
635 concentrations. Values were produced as a mean of 3 independent experiments performed in
636 duplicate.
637

638 **Figure 1: Checkerboard assessment of synergy between HT61 and tobramycin**

639 Synergy as shown in an example (strain RP73) of the checkerboard assay (A) and the mean
640 FICIs for the three strains tested (B). Each plate contained two control wells; a negative
641 (inhibitor on its own) and a positive control (no inhibitor). Negative controls or NC,
642 containing no bacterial cells, were also included. Resazurin was used to determine the
643 growth-no growth interface as shown by pink wells (alive and active cells) or blue wells (no
644 dye conversion). MICs for agents alone or in combination were determined and used to
645 calculate FICIs (B). FICIs are shown as the mean of 5 independent experiments, bar
646 represents SEM.

647

648 **Figure 2: Time kill assay demonstrating synergy between tobramycin and HT61 against**
649 ***P. aeruginosa.***

650 Effect of sub-inhibitory concentrations of tobramycin (•), HT61 (■) and combination (▲) on
651 the viability of *P. aeruginosa* strains PAO1 (A), RP73 (B) and NN2 (C). Samples were taken

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652 for enumeration at 0, 4, 8, 12 and 24 hours and number of live bacterial cells were plotted as
653 $\log_{10}$. (CFU/mL). Results are the mean of two independent experiments, bars show SEM.

654

655 **Figure 3: HT61 reduces the tobramycin concentration required to eradicate established**
656 **biofilm (MBEC)**

657 Effect of fixed doses of HT61 (25, 50 or 100 µg/mL) on tobramycin MBEC on *P. aeruginosa*
658 strain PAO1 (A), NN2 (B) or RP73 (C). MBEC were determined as the lowest concentration
659 of tobramycin to inhibit bacterial recovery after antibacterial challenge of established
660 biofilms. Data are expressed as mean of MBEC (n = 9-12), bars represent SEM. * P<0.05 and
661 **** P<0.0001 non-treated control vs HT61 at 25, 50 or 100 µg/mL (unpaired student t-test
662 with Welch's correction).

663

664 **Figure 4: Effect of single treatment of HT61 against *P. aeruginosa* in a murine model of**
665 **pulmonary infection**
666 Mice were infected with either sterile PBS embedded agar beads, $1x10^6$ cfu/mouse *P.*
667 *aeruginosa* strains RP73 (**A**), NN2 (**B**) or PA01 (**C**), embedded agar beads, via *o.a.*
668 inoculation. 24 hours post infection, mice were administered with either vehicle, 0.1, 1 and 5
669 mg/kg HT61 via intraperitoneal injection. 48 hours post infection, colony forming units were
670 quantified in lung homogenate on TSA plates. n=3-5, data expressed as log mean ± SEM. ***
671 P< 0.001 versus sham control mice. LOD = Limit of Detection. Data analysed using one-way
672 ANOVA and Sidak's multiple comparisons post-test.
673
674
675 **Figure 5: Effect of combination treatment of 1mg/kg HT61 and tobramycin against *P.***
676 ***aeruginosa* in a murine model of pulmonary infection**
677 Mice were infected with either sterile PBS embedded agar beads, $1x10^6$ cfu/mouse *P.*
678 *aeruginosa* strains RP73 **(A)**, NN2 **(B)**, PA01 **(C)** embedded agar beads, via *o.a.* inoculation.
679 24 hours post infection, mice were administered with either vehicle, tobramycin (100mg/kg)
680 and HT61 (1 mg/kg) as single treatments, or combination treatments 100 mg/kg tobramycin +
681 1 mg/kg HT61 via intraperitoneal injection. 48 hours post infection, colony forming units
682 were quantified in lung homogenate on TSA plates. n=4-5, data expressed as log mean ±
683 SEM. ### P< 0.001 versus vehicle, # P<0.05 versus vehicle. LOD = Limit of Detection. Data
684 analysed using one-way ANOVA and Sidak's multiple comparisons post-test.
685
686
687 **Figure 6: Effect of combination treatment of 10mg/kg HT61 and tobramycin against *P.***
688 ***aeruginosa* in a murine model of pulmonary infection**
689 Mice were infected with either sterile PBS embedded agar beads, $1x10^6$ cfu/mouse *P.*
690 *aeruginosa* strains RP73 **(A)**, NN2 **(B)** or PA01 **(C)** embedded agar beads, via *o.a.*
691 inoculation. 24 hours post infection, mice were administered with either vehicle, tobramycin
692 (100mg/kg) and HT61 (10 mg/kg) as single treatments, or combination treatments 100 mg/kg
693 tobramycin + 10 mg/kg HT61 via intraperitoneal injection. 48 hours post infection, colony
694 forming units were quantified in lung homogenate on TSA plates. n=4-5, data expressed as
695 log mean ± SEM. versus sham control mice, ### P< 0.001 versus vehicle, # P<0.05 versus
696 vehicle, δ P<0.05 vs 1mg/kg HT61. LOD = Limit of Detection. Data analysed using one-way
697 ANOVA and Sidak's multiple comparisons post-test.

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700 **Supplementary Table 1: MICs values of tobramycin and HT61 alone or in combination**
701 **and corresponding FICs**
702 Data are the mean of 5 independent experiments ± SEM from checkerboard assay
703

704 **Supplementary Table 2:** Predicted and experiment reductions in bacterial numbers (log
705 cfu/mL) recovered from a murine model of pulmonary infection with either the *P. aeruginosa*
706 strains RP73, NN2 and PA01. Predicted additive reduction was calculated by adding the
707 reduction of HT61 and Tobramycin alone for each strain, this was then compared against the
708 reduction in bacterial cell numbers following combination treatment
709

710
711
712 **Supplementary Figure 1: Tobramycin dose response curves**
713 Mice were infected with either sterile PBS embedded agar beads, $1x10^6$ cfu/mouse *P.*
714 *aeruginosa* strains RP73 **(A)**, NN2 **(B)** or PA01 **(C)** embedded agar beads, via *o.a.*
715 inoculation. 24 hours post infection, mice were administered with vehicle or increasing
716 concentrations of tobramycin. 48 hours post infection, colony forming units were quantified
717 in lung homogenate on TSA plates. n=4-5, data expressed as log mean ± SEM. versus sham
718 control mice, ### P< 0.001 versus vehicle, # P<0.05 versus vehicle, δ P<0.05 vs 1mg/kg
719 HT61. LOD = Limit of Detection. Data analysed using one-way ANOVA and Sidak's
720 multiple comparisons post-test.
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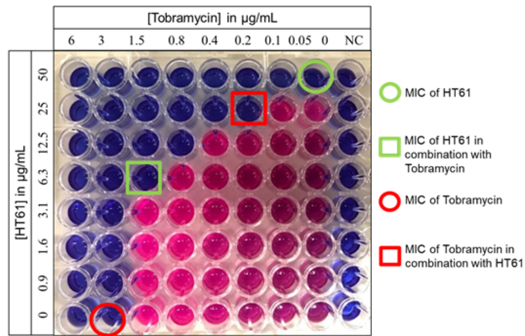
Figure 1

A



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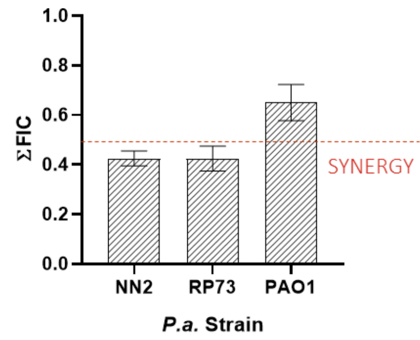

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Figure 2

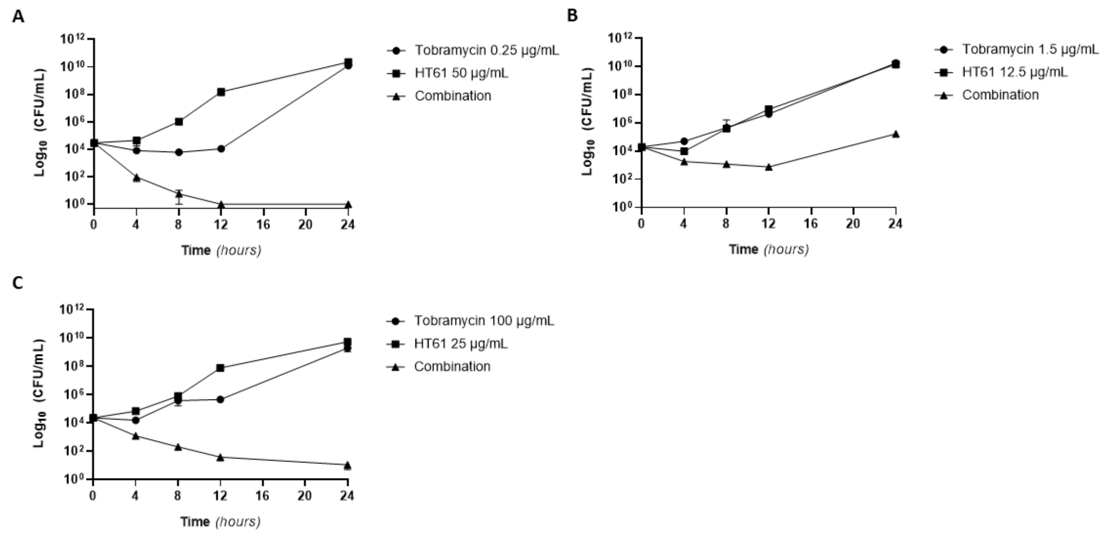

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Figure 3

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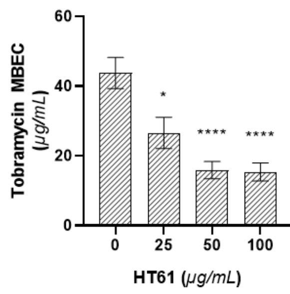

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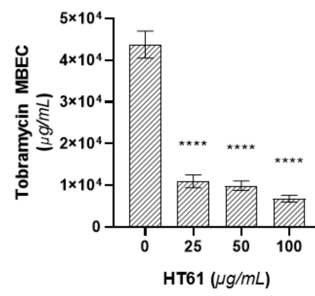

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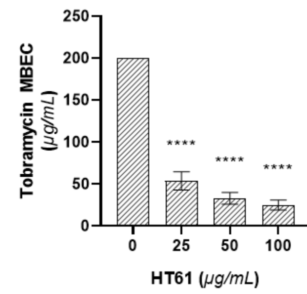

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Figure 4

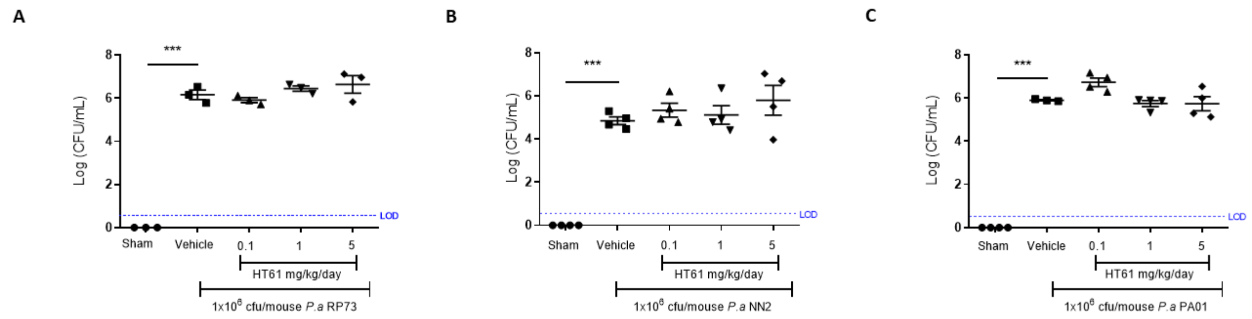

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Figure 5

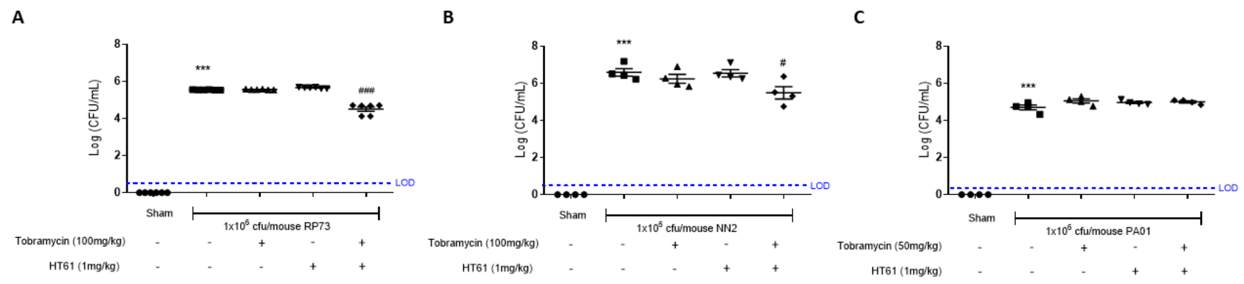

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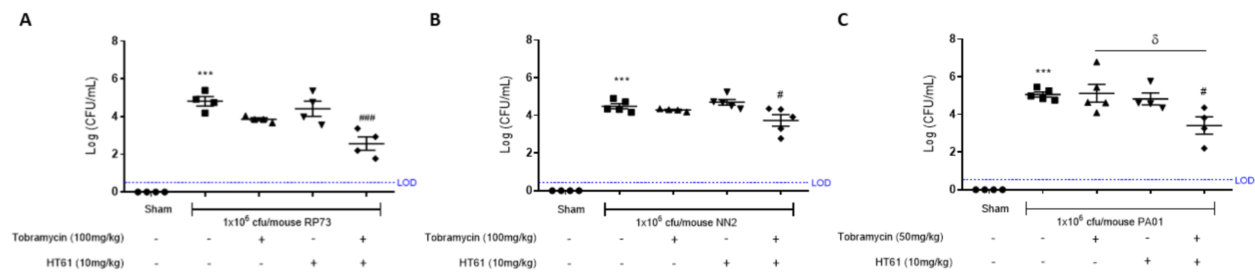
Figure 6



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