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PII: S1094-5539(19)30321-9
DOI: https://doi.org/10.1016/j.pupt.2019.101884
Reference: YPUPT 101884

To appear in: Pulmonary Pharmacology & Therapeutics

Received Date: 24 December 2019
Accepted Date: 25 December 2019

Please cite this article as: Amison RT, Faure M-E, O'Shaughnessy BG, Bruce KD, Hu Y, Coates A, Page CP, The small quinolone derived compound HT61 enhances the effect of tobramycin against Pseudomonas aeruginosa in vitro and in vivo, Pulmonary Pharmacology & Therapeutics (2020), doi: https://doi.org/10.1016/j.pupt.2019.101884.

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


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Running Title: HT61 enhances the antimicrobial activity of tobramycin

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

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

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

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

The small quinolone-derived compound HT61, is one such molecule that has previously demonstrated synergy with the aminoglycoside antibiotics neomycin and gentamicin. In these studies, synergy was observed against the gram-positive bacteria MRSA and MSSA *in vitro* and in an *in vivo* model of murine skin infection (10). Whilst HT61 has been shown to enhance the activity of some antibiotics, it has also demonstrated direct bactericidal activity against gram-positive bacteria (11). This bactericidal activity has been proposed to be a result of the cationic charge of HT61 partitioning into the negatively charged bilayer of the bacterical cell membrane causing structural changes resulting in a loss of membrane integrity and depolarisation. Ultimately, this severe
membrane damage induces the expulsion of intracellular components and eventually, cell death (12,13). This direct bactericidal activity of HT61 has previously been used as a topical agent and continues to advance through clinical trials with the ultimate aim of decolonising S. aureus from the nasal cavity (14).

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

2.1. Bacterial Culture conditions and maintenance

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

2.2. Chemicals used in this study

All chemicals were purchased from Sigma Aldrich (Sigma-Aldrich, Dorset, UK) unless stated otherwise. All *in vitro* materials were sterilised by autoclaving at 121°C for 20 minutes. HT61-HCl was generously donated by Professor Sir Anthony Coates, St Georges Hospital, London and Helperby Therapeutics, and first dissolved in DMSO (10 g/L). Stock solutions were prepared by the addition of dH$_2$O to 2.4 g/L prior to sterilisation as effected by 0.22μm filtration (Thermo Fisher Scientific, Loughborough, UK). HT61 solutions were stored at -20°C. Tobramycin stocks were made in dH$_2$O to give 10 g/L or 50 g/L working concentrations that were sterilised by using filtration at 0.22 μm. Solutions were stored at -20°C.
2.3. Minimum Inhibitory Concentration (MIC) Assay

MICs were determined according to the protocols described previously (18). Serial twofold dilutions of tobramycin and HT61 were prepared in distilled water in flat well polycarbonate 96 well plates (Greiner Bio-One Ltd, Stonehouse, UK). To each well, double strength CAMH broth was added. Colonies from overnight incubation on CAMH agar were resuspended in sterile saline and c. $5 \times 10^5\, \text{cfu/mL}$ were inoculated. Plates were sealed with a “breathable membrane” (Greiner Bio-One Ltd) and incubated in a wet chamber at 37°C for 16-20 hours. Bacterial load was measured by optical density at 600 nm by using a Fluostar Omega reader (BMG Labtech, Ortenberg, Germany) and used to plot a concentration response curve.

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

2.4. Synergy assessment by Checkerboard assay

The chequerboard methods was used for the measurement of potential synergy from combinations of HT61 with tobramycin against each of the 3 strains of *P. aeruginosa* as previously described (19,20). Briefly, the combinations of Tobramycin and HT61 were prepared using 96-well plates using drugs concentrations starting from 2 fold higher than their MIC values and the serially diluting 2-fold to zero in sterile flat well polycarbonate plates (Greiner Bio-One Ltd) to create 64 combinations. The
experiment was then performed using the MIC methodology as described above. The growth/no
growth interface was then determined using the visual colour change of resazurin (blue) in to the
pink resorufin product and used to plot isobolograms and for the calculation of the fractional
inhibitory concentration (FIC) as a measure of the effects of combination therapies.

The sum of FICs or FIC indexes (FICIs) were calculated as $\Sigma \text{FIC}_{A+B} = \text{FIC}_A + \text{FIC}_B$ where $\text{FIC}_A = \frac{\text{MIC}_{A+B}}{\text{MIC}_A}$ and $\text{FIC}_B = \frac{\text{MIC}_{A+B}}{\text{MIC}_B}$.

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

2.5. Synergy assessment by Bacterial Time Kill Assays

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

2.6. Minimum biofilm concentration eradication assay

Effect of combination therapy was studies on biofilms by using the Calgary devices as previously
described with minor modifications (24). One colony was inoculated in CAMH and incubated (37°C,
130 rpm). After approximately 20 hours, cells were washed twice by centrifugation (5,000g, 4°C) and
resuspension in sterile 0.9% NaCl. Bacterial suspensions were diluted to $10^5$ cfu/mL in CAMH and inoculated in a 96 well plate (150 μL per wells). Plate lids were replaced with the Nunc-TSP 96 Pin lid and biofilms were left to form for 18-20 hours under shaking (110 rpm) in a wet atmosphere. Established biofilms on pins were washed with 200 μL of sterile 0.9% NaCl and transferred into a challenge plate. Challenge plates were made of serial dilutions of tobramycin supplemented with HT61 at 25, 50 and 100 μg/mL in CAMH (200 μL per wells). For each plate, controls of tobramycin and HT61 alone were present, as well as sterility controls. Biofilms were challenged for 18-20 hours under the same condition (37°C, 110 rpm, wet atmosphere). Following challenge, biofilms were placed in an inhibitor free environment (CAMH, 200 μL per wells), left to equilibrate at room temperature for 30 minutes and sonicated at high intensity for 30 minutes. Sonication efficiency were visually checked by crystal violet 0.1 % staining. Recovery plates containing detached biofilms were incubated for a further 24 to 48 hours and used to determine MBEC (minimal biofilm eradication concentration), where an MBEC was defined as the lowest concentration to inhibit growth as previously recommended.

2.7. Murine Model of Pulmonary Infection

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

Bacterial embedded agar beads were prepared using the protocol described previously (25). Briefly, bacterial cultures were prepared by inoculating a TSA plate with a stock cryobead and incubating at 37°C for 24 hours under static conditions. 24 hours prior to use, secondary overnight cultures were prepared by inoculating 20ml of TSB with 2-3 bacterial colonies and subsequently incubated at 37°C for 16 hours under rotary conditions of 120rpm. Bacteria were embedded into agar beads by mixing the secondary overnight culture with molten TSA at a 1:9 ratio, which was then spun into mineral oil,
previously heated to 50°C. The preparation was cooled and centrifuged at 2700 g and the remaining mineral oil was removed by washing with sterile PBS. Bacterial content of the beads was then quantified on TSA plates and diluted to achieve $2 \times 10^7$ cfu/mL using sterile PBS, to deliver a final dose of $1 \times 10^6$ cfu/mouse.

Animals were housed in filter top cages under standard conditions of 22±2°C with a 12:12 light: dark cycle. All animals were provided with food and water \textit{ad libitum}, and wood shavings, shredded paper and cardboard tubes were provided for environmental enrichment. All animals were provided with a minimum acclimatization period of 7 days upon arrival before the commencement of the study. Male C57/Bl6J mice (8 weeks, Harlan, United Kingdom) were anesthetized with inhaled isoflurane and inoculated with $1 \times 10^6$ cfu/mouse bacterial embedded agar beads via oropharyngeal infection (o.a.). Sham mice were inoculated with sterile PBS embedded agar beads via the o.a. route of administration, as a control. Animals were weighed and monitored daily for signs of pain and distress. Animals that lost more than 20% of their body weight throughout the duration of the study were euthanised. 24 hours post infection, mice were treated with either saline vehicle, HT61, Tobramycin or a combination treatment of HT61 and Tobramycin via intraperitoneal (i.p.) injection. 48 hours post infection, lungs were aseptically removed and homogenized in 2mL sterile saline. Serial dilutions of lung homogenates were performed, and appropriate dilutions plated onto TSA plates and incubated for 24 hours under static conditions. Colonies were manually quantified and log (CFU/mL) calculated.

2.8. Statistical Analysis

Chequerboard analysis of synergy was used to identify a fractional inhibitory concentration (FIC) index, where synergy was determined if the FIC was ≤0.5. Additivity or no interaction was described if the FIC was >0.5 but <4.0 and antagonism if the FIC was >4.0. \textit{In vitro} Time-kill analyses of data
were presented as mean ± standard error of the mean (SEM) and analysed by using GraphPad Prism 8.0. Due to experimental variation, MBEC were expressed as mean of 9 to 12 replicates. These data were normally distributed as shown by the QQ plots in S2 Fig. They were therefore eligible for parametric testing. Differences between untreated control and each of the treated groups (25, 50 or 100 µg/mL) were analysed by using an unpaired student t-test with Welch’s correction. The one-way analysis of variance and Sidak’s multiple comparisons post-test were used for comparisons of data. All in vivo data are presented as mean ± standard error of the mean of log cfu/ml and analysed using GraphPad Prism 8.0. The one-way analysis of variance and Sidak’s multiple comparisons post-test were used for comparisons of data. P values of less than 0.05 were determined to demonstrate a significant difference.

3. Results

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

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

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

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

The bactericidal activities of HT61 and tobramycin in combination were assessed in bacterial time-kill assays over a 24 hours timescale. Fig 2 shows the time kill assay data obtained for *P. aeruginosa* strains PAO1 (A), RP73 (B) and NN2 (C). HT61 (black squares) and tobramycin (black circles) were used at subinhibitory concentrations, which did not significantly impact the final number of *P. aeruginosa* cells (growth to approximately 10^10 CFU/mL for all strains examined). Assays that
combined both agents at the same concentration are shown as black triangles. Synergy was subsequently defined as when the difference in bacterial numbers exceeded two log orders, when comparing a combination of agents to single agents. Strong evidence for synergy was identified after eight hours for strain PAO1 (Fig 2A), with no cfu’s detected for PA01 after 12 hours following combination treatment of HT61 and Tobramycin in comparison to $10^8$ cfu/ml following HT61 monotherapy and $10^4$ cfu/ml following Tobramycin monotherapy at the same timepoint.

For strain RP73 (Fig 2B), no difference between HT61 and Tobramycin monotherapies were observed at any timepoint. However, combination therapy of HT61 and Tobramycin demonstrated a 2 log fold reduction in recovered bacterial cfu at 8 hours (HT61 Monotherapy: 4.65x$10^5$ cfu/ml; Tobramycin Monotherapy: 3.93x$10^5$ cfu/ml; Combination therapy: 1.2x$10^3$ cfu/ml) indicating synergy between the two compounds. The same trend was also observed for the Tobramycin resistant strain NN2 (Fig 2C), where no difference between HT61 and Tobramycin monotherapies were observed, whilst combination therapy of HT61 and Tobramycin once again demonstrated a 3 log fold reduction in recovered bacterial cfus at 8 hours (HT61 Monotherapy: 3.89x$10^5$ cfu/ml; Tobramycin Monotherapy: 8x$10^5$ cfu/ml; Combination therapy: 2x$10^2$ cfu/ml) from 8 hours onwards.

3.4. HT61 significantly enhances tobramycin mediated biofilm eradication

Since *P. aeruginosa* infections are typically associated with bacteria persisting as biofilms, the impact of combinations of HT61 and tobramycin was also investigated on these structured forms of growth. Firstly, we investigated whether biofilm establishment *in vitro* was affected by singular treatment with either HT61 or tobramycin through the checkerboard assay system. In our studies we observed similar FICs values similar to those obtained from produced in the non-biofilm grown samples(data not shown).
Secondly, we aimed to investigate the effect of tobramycin and HT61 on already established biofilms. Biofilms, formed in inhibitor free conditions, were challenged with tobramycin and HT61. The period of recovery required was used to determine the minimum biofilm eradication concentration (MBEC). HT61 given as a monotherapy had no impact on bacterial cell numbers in previously established biofilm structures (data not shown). As such, the impact of different concentrations of tobramycin in the presence of fixed levels of HT61 (25, 50 or 100 μg/mL) was assessed (Fig 3). For all strains tested, the addition of HT61 significantly enhanced the ability of tobramycin to eradicate biofilm located cells, as shown by the decreased MBEC. For strain PAO1 (Fig 3A), the lowest concentration of tobramycin required to eradicate biofilms was 43.75 ± 4.49 μg/mL. The addition of 25, 50 or 100 μg/mL of HT61 significantly reduced this MBEC to 26.56 ± 4.49 μg/mL (p<0.05), 15.89 ± 2.48 μg/mL and 15.36 ± 2.59 μg/mL respectively (p<0.0001). For strain NN2 (Fig 3B), MBEC for tobramycin alone was above 25 g/L. As the solubility limit was reached, tobramycin MBEC was assumed to be 50 g/L for those replicates. The tobramycin MBEC alone was therefore estimated at 43.75 ± 3.26 g/L, which was significantly reduced (p<0.0001) by at least 4-fold when 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 enhancement on biofilm eradication was observed with RP73 (Fig 3C). When used as a single agent, 200 μg/mL tobramycin was required to eradicate RP73 biofilm structures, whilst HT61 at 25, 50 or 100 μg/mL was shown to lower the tobramycin MBEC to 53.75 ± 10.87 μg/mL (3.7 fold), 33.13 ± 6.91 μg/mL (6-fold) and 25.0 ± 6.04 μg/mL (8-fold) respectively.

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

The *in vitro* data described in sections 3.1-3.4 described an ability of HT61 to enhance the activity of tobramycin against both RP73 and NN2. We therefore investigated whether this enhancement was replicated in *in vivo* studies using a murine model of pulmonary infection with *P. aeruginosa*. Dose-response titrations of HT61 were performed towards infections with all three strains of *P.
aeruginosa described above, in order to determine whether HT61 monotherapy demonstrated any bactericidal activity in vivo. In all studies, inoculation with $1 \times 10^6$ cfu RP73/NN2/PA01 per mouse resulted in significant increases in bacterial cell numbers when compared to sham controls (Sham: $0.00 \pm 0.00$ log cfu/ml vs. RP73: $6.16 \pm 0.22$ log cfu/ml, $P < 0.001$; NN2: $4.85 \pm 0.18$ log cfu/ml, $P < 0.001$; PA01: $5.90 \pm 0.03$ log cfu/ml, $P < 0.001$, Figs 4A-C). Single systemic treatment with 0.1, 1 and 5mg/kg HT61 i.p. failed to demonstrate any reduction in pulmonary bacterial load when compared to vehicle treated controls at 48 hours against any of the three P. aeruginosa strains tested; RP73 (Vehicle: $6.16 \pm 0.22$ log cfu/ml vs 0.1mg/kg: $5.91 \pm 0.12$ log cfu/ml; 1mg/kg: $6.45 \pm 0.12$ log cfu/ml; 5mg/kg: $6.64 \pm 0.41$ log cfu/ml), NN2 (Vehicle: $4.85 \pm 0.18$ log cfu/ml vs 0.1mg/kg: $5.34 \pm 0.32$ log cfu/ml; 1mg/kg: $5.12 \pm 0.43$ log cfu/ml; 5mg/kg: $5.80 \pm 0.69$ log cfu/ml) or PA01 (Vehicle: $5.90 \pm 0.03$ log cfu/ml vs 0.1mg/kg: $6.73 \pm 0.20$ log cfu/ml; 1mg/kg: $5.74 \pm 0.14$ log cfu/ml; 5mg/kg: $5.74 \pm 0.33$ log cfu/ml). This suggested that unlike in previous studies performed against gram-positive bacteria, HT61 has no direct anti-bacterial activity against gram-negative P. aeruginosa.

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

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

Initial dose-response experiments were performed to identify a sub-threshold dose of tobramycin with which failed to reduce bacterial numbers retrieved from the lungs 48 hours post infection when compared to vehicle controls (Supplementary Figure 1). From these experiments, 100mg/kg
systemic tobramycin was selected for the drug resistant *P. aeruginosa* strains RP73 and NN2, whilst 50mg/kg was selected for *P. aeruginosa* reference strain PA01.

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

Next, we investigated whether the inability of HT61 to potentiate the activity of tobramycin against PA01 was due to an insufficient dose of HT61 used in the combination treatments. We therefore replicated the above studies using an increased 10mg/kg HT61 in the combination treatment with tobramycin. With this increased dose of HT61, we observed significant reductions in pulmonary bacterial numbers against vehicle control mice 48 hours post infection with all strains, RP73 (Vehicle: 4.81 ± 0.25 log cfu/ml vs 10mg/kg HT61 + 100mg/kg tobramycin: 2.57 ± 0.36 log cfu/ml, *P* < 0.001),
NN2 (Vehicle: 4.49 ± 0.14 log cfu/ml vs 10mg/kg HT61 + 100mg/kg tobramycin: 3.74 ± 0.31 log cfu/ml, P < 0.05) and PA01 (Vehicle: 4.96 ± 0.11 log cfu/ml vs 10mg/kg HT61 + 50mg/kg tobramycin: 3.41 ± 0.46 log cfu/ml, P < 0.05) (Figs 6A-C).

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

4. Discussion

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

In this study, 3 different strains of *P. aeruginosa* were considered including the multi-drug resistant (RP73) and tobramycin resistant (NN2) strains (15) alongside PAO1 as a reference laboratory strain.
As such, these strains represented a range of known phenotypic responses to tobramycin. The observation that HT61 in combination with tobramycin produced a synergistic effect in vitro against the strains considered as resistant to tobramycin was therefore encouraging. In turn, this provided a strong rationale for the assessment of the antibacterial activity of combinations of HT61 and tobramycin in vivo using a murine model of pulmonary infection. As such, our observations have extended previous work with HT61 where this small quinolone derived compound has demonstrated activity against gram-positive species, including *Staphylococcus aureus* (both MSSA and MRSA) in vitro and in a wound infection model (10,11,26,27). Importantly, in these studies HT61 was also observed to enhance the bactericidal activity of existing antibiotics including gentamicin and neomycin against both MSSA and MRSA in addition to its own individual bactericidal capabilities (11,28). Extending from these studies our focus here was on *P. aeruginosa*, which demonstrates significant resistance to current therapy. The finding in this study that both a tobramycin resistant and multi-drug resistant strain of this species were rendered sensitive – as if “restored” – was therefore a novel and exciting finding and the potential clinical benefits of such a finding are clear. Through the identification of the requirement of HT61 in this combinational role, we propose this class of antibacterial agent as an enhancer of the efficacy and impact of conventional antibiotics, and therefore propose the definition of ‘antibiotic enhancer’ as a substance that in relatively low concentration extends the antibacterial activity of a conventional antibiotic, despite having no or low-level antibacterial activity in itself.

Given the concern over the lack of novel antibiotics in the pipeline, alternate strategies designed to maximise and enhance the activity and in turn longevity of existing antibiotics are important. Borrowing the same terminology as for antibiotic susceptibility, enhancers could either be narrow or broad in their spectrum. The ability of HT61 therefore to render RP73, a nearly tobramycin resistant strain (MIC of 3µg/mL), susceptible to tobramycin therapy (MIC reduced to 0.6µg/mL), both in vitro and in vivo is therefore highly important. Such enhancer strategies would avoid scenarios whereby resistance to an antibiotic is overcome by simply increasing the dose of the antibiotic prescribed.
This is of particular relevance for aminoglycosides such as tobramycin, as it is neither feasible or ethical to significantly increase the doses used due to their systemic toxicity profiles, as in addition to their ototoxic and nephrotoxic side effects, chronic kidney disease has also been associated with chronic tobramycin treatment, in individuals with cystic fibrosis (29–32). As an alternative to simply increasing antibiotic plasma levels, the use of multiple antibiotics in combination has been proposed as a strategy capable of slowing the emergence of antimicrobial resistance whilst also shortening the required duration of therapy (33). Antibiotics from a number of different classes have previously been reported to show synergistic effects against tobramycin resistant strains of *P. aeruginosa* (34), therefore enhancer strategies (or antibiotic resistance breakers), featuring low or no direct antibiotic impact, may therefore be less susceptible as a target for resistance emergence and could be a viable alternative approach (35,36).

Resistance, emerging or intrinsic, is a feature of the pathogen *P. aeruginosa* (37). Despite the layered nature of the resistance characteristic of *P. aeruginosa*, compounds able to compromise the integrity of bacterial cell membranes are likely to enhance antibiotic penetration. The mechanism by which HT61 may enhance the activity of classical antibiotics through its non-specific targeting the anionic lipids in the bacterial membrane due to the negative charge of HT61. In the case of *S. aureus* such anionic lipids may include phosphatidylglycerol (PG) and the zwitterionic phosphatidylethanolamine (12,13). In targeting these lipids, HT61 induces rapid partitioning of the lipid bilayer into a monolayer causing structural changes thus impairing membrane integrity leading to depolarization and catastrophic membrane damage (12,13). In contrast to previous studies, where HT61 has shown bactericidal activity against strains of *S. aureus* (10,11), HT61 treatment as a monotherapy had little or no bactericidal effects on any of the tested strains of *P. aeruginosa* in any of our *in vitro* or *in vivo* models. This may be due to differences in the membrane lipid composition between the two species (38,39) with cationic membrane acting agents such as HT61 showing greater activity against *S. aureus* (40).
Using a pharmacodynamic model, Bulitta et al. accounted for two killing mechanisms associated with aminoglycoside use against *P. aeruginosa* (41). Delayed killing was attributed to the effect of tobramycin on bacterial protein synthesis whilst immediate killing was attributed to disruption of the outer membrane (41). It is possible that our observation of increased tobramycin activity when combined with HT61 is as a result of the initial disruption of the outer membrane by tobramycin increasing the exposure of the anionic phospholipids on the inner cytoplasmic membrane to HT61. The resulting disruption of both membranes would increase the permeability of the gram-negative bacterial membranes to tobramycin, resulting in an elevated intracellular tobramycin concentration enhancing the bactericidal activity through its effects on protein synthesis (10,11). Whilst the mechanism of action of HT61 remains open for discussion and further studies are required to fully understand the mechanism of action of HT61 in the potentiation of tobramycin against gram-negative bacteria, it is also important to consider the impact on the mode of growth of *P. aeruginosa*. Here, the impact on biofilm formation, as well as the impact of HT61/tobramycin on existing biofilms was also examined. Whilst HT61 had no impact on either establishing or established biofilm structures in our tested concentration range, when treated in combination with tobramycin we demonstrated a 10-fold reduction in the MBEC observed with tobramycin alone.

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

In conclusion, the combination of tobramycin with HT61 demonstrated significant potentiation of bactericidal activity when compared to tobramycin as a monotherapy against both tobramycin sensitive and resistant strains of *P. aeruginosa*, in both *in vitro* assays and an *in vivo* model of lung infection. This indicates the potential benefits of combination treatments using enhancer
compounds, such as HT61 alongside conventional antibiotics including tobramycin in the treatment of antibiotic resistant gram-negative infections. Such enhancer strategies would support the use of lower doses of the aminoglycoside antibiotics significantly reducing their associated toxicity profiles thus providing a potentially novel way of targeting the ongoing global issue of antimicrobial resistance.

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

6. References


**Table 1: *P. aeruginosa* susceptibility profile to tobramycin and HT61**

<table>
<thead>
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<th><em>P. aeruginosa</em> strain</th>
<th>Antibiotic resistance</th>
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<td>PAO1</td>
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MIC values derived from dose-response curve data for HT61 and tobramycin with three strains of *P. aeruginosa* PAO1, RP73 and NN2. Here MIC values are displayed as µg/ml concentrations. Values were produced as a mean of 3 independent experiments performed in duplicate.

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

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

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

Effect of sub-inhibitory concentrations of tobramycin (•), HT61 (■) and combination (▲) on the viability of *P. aeruginosa* strains PAO1 (A), RP73 (B) and NN2 (C). Samples were taken.
for enumeration at 0, 4, 8, 12 and 24 hours and number of live bacterial cells were plotted as \( \log_{10} \) (CFU/mL). Results are the mean of two independent experiments, bars show SEM.

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

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

**Figure 4: Effect of single treatment of HT61 against *P. aeruginosa* in a murine model of pulmonary infection**

Mice were infected with either sterile PBS embedded agar beads, \( 1 \times 10^6 \) cfu/mouse *P. aeruginosa* strains RP73 (A), NN2 (B) or PA01 (C), embedded agar beads, via *o.a.* inoculation. 24 hours post infection, mice were administered with either vehicle, 0.1, 1 and 5 mg/kg HT61 via intraperitoneal injection. 48 hours post infection, colony forming units were quantified in lung homogenate on TSA plates. n=3-5, data expressed as log mean ± SEM. *** P< 0.001 versus sham control mice. LOD = Limit of Detection. Data analysed using one-way ANOVA and Sidak’s multiple comparisons post-test.

**Figure 5: Effect of combination treatment of 1mg/kg HT61 and tobramycin against *P. aeruginosa* in a murine model of pulmonary infection**

Mice were infected with either sterile PBS embedded agar beads, \( 1 \times 10^6 \) cfu/mouse *P. aeruginosa* strains RP73 (A), NN2 (B), PA01 (C) embedded agar beads, via *o.a.* inoculation. 24 hours post infection, mice were administered with either vehicle, tobramycin (100mg/kg) and HT61 (1 mg/kg) as single treatments, or combination treatments 100 mg/kg tobramycin + 1 mg/kg HT61 via intraperitoneal injection. 48 hours post infection, colony forming units were quantified in lung homogenate on TSA plates. n=4-5, data expressed as log mean ± SEM. ### P< 0.001 versus vehicle, # P<0.05 versus vehicle. LOD = Limit of Detection. Data analysed using one-way ANOVA and Sidak’s multiple comparisons post-test.

**Figure 6: Effect of combination treatment of 10mg/kg HT61 and tobramycin against *P. aeruginosa* in a murine model of pulmonary infection**

Mice were infected with either sterile PBS embedded agar beads, \( 1 \times 10^6 \) cfu/mouse *P. aeruginosa* strains RP73 (A), NN2 (B) or PA01 (C) embedded agar beads, via *o.a.* inoculation. 24 hours post infection, mice were administered with either vehicle, tobramycin (100mg/kg) and HT61 (10 mg/kg) as single treatments, or combination treatments 100 mg/kg tobramycin + 10 mg/kg HT61 via intraperitoneal injection. 48 hours post infection, colony forming units were quantified in lung homogenate on TSA plates. n=4-5, data expressed as log mean ± SEM. versus sham control mice, ### P< 0.001 versus vehicle, # P<0.05 versus vehicle, δ P<0.05 vs 1mg/kg HT61. LOD = Limit of Detection. Data analysed using one-way ANOVA and Sidak’s multiple comparisons post-test.
Supplementary Table 1: MICs values of tobramycin and HT61 alone or in combination and corresponding FICs
Data are the mean of 5 independent experiments ± SEM from checkerboard assay

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

Supplementary Figure 1: Tobramycin dose response curves
Mice were infected with either sterile PBS embedded agar beads, 1x10^6 cfu/mouse \textit{P. aeruginosa} strains RP73 (A), NN2 (B) or PA01 (C) embedded agar beads, via o.a. inoculation. 24 hours post infection, mice were administered with vehicle or increasing concentrations of tobramycin. 48 hours post infection, colony forming units were quantified in lung homogenate on TSA plates. n=4-5, data expressed as log mean ± SEM, versus sham control mice, ### P< 0.001 versus vehicle, # P<0.05 versus vehicle, δ P<0.05 vs 1mg/kg HT61. LOD = Limit of Detection. Data analysed using one-way ANOVA and Sidak’s multiple comparisons post-test.
Figure 2

A

- Tobramycin 0.25 µg/mL
- HT61 50 µg/mL
- Combination

B

- Tobramycin 1.5 µg/mL
- HT61 12.5 µg/mL
- Combination

C

- Tobramycin 100 µg/mL
- HT61 25 µg/mL
- Combination
Figure 5

A

B

C

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Figure 6

A

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