A *Galleria mellonella* infection model reveals double and triple antibiotic combination therapies with enhanced efficacy versus a multidrug-resistant strain of *Pseudomonas aeruginosa*

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The aim of this study was to compare the inhibitory effect of antibiotic combinations *in vitro* with efficacy in *Galleria mellonella* larvae *in vivo* to identify efficacious combinations that target *Pseudomonas aeruginosa*. *P. aeruginosa* NCTC 13437, a multidrug-resistant strain resistant to β-lactams and aminoglycosides, was used. Susceptibility to cefotaxime, piperacillin, meropenem, amikacin, levofloxacin and colistin alone, or in dual or triple combinations, was measured *in vitro* via a 24 h time-kill assay. *In vitro* results were then compared with the efficacy of the same dual or triple antibiotic combinations versus *G. mellonella* larvae infected with *P. aeruginosa*. *G. mellonella* haemolymph burden of *P. aeruginosa* was determined over 96 h post-infection and treatment with the most potent combination therapies. Many dual and triple combinations of antibiotics displayed synergistic inhibition of multidrug-resistant *P. aeruginosa* *in vitro*. There was little correlation between combinations that were synergistic *in vitro* and those that showed enhanced efficacy *in vivo* versus infected *G. mellonella* larvae. The most potent dual and triple combinations *in vivo* were cefotaxime plus piperacillin, and meropenem plus piperacillin and amikacin, respectively. Fewer combinations were found to offer enhanced therapeutic benefit *in vivo* compared with *in vitro*. The therapeutic benefit arising from treatment with antibiotic combinations *in vivo* correlated with reduced larval burden of *P. aeruginosa*. This study has identified antibiotic combinations that merit further investigation for their clinical potential and has demonstrated the utility of using *G. mellonella* to screen for novel antibiotic treatments that demonstrate efficacy *in vivo.*

**INTRODUCTION**

The majority of life-threatening infections caused by *Pseudomonas aeruginosa* are hospital-acquired due to increased colonization rates as a consequence of invasive procedures (e.g. catheterization and mechanical ventilation), greater likelihood of patients being immunocompromised, and broad-spectrum antimicrobial treatment having a detrimental effect on the normal flora (Lister et al., 2009). Thus, *P. aeruginosa* is a frequent cause of nosocomial pneumonia, urinary tract infections, surgical site infections and bacteraemia particularly in the intensive care unit (Spencer, 1996; Lister et al., 2009). Compounding the clinical problem of *P. aeruginosa* infection is the ease with which the organism acquires resistance to multiple antibiotics. The incidence of isolation of multidrug-resistant (MDR) strains (defined as resistant to three or more classes of antibiotics) is increasing. For example, a study of 37 000 *P. aeruginosa* isolates from intensive care units in the USA reported an increase in the proportion of MDR strains from 13 % to 21 % over the period 1997–2002 (Livermore, 2002). The rising incidence of MDR strains of *P. aeruginosa* complicates the therapy of infections that they cause because the immediate administration of an appropriate antibiotic therapy is a major factor determining a favourable outcome for the patient (Micek et al., 2005). For example, infection with MDR *P. aeruginosa* was associated with increased mortality, hospital stay and requirement for surgical procedures (Aloush et al., 2006).

In an effort to improve therapeutic outcome, many clinicians have attempted to treat MDR *P. aeruginosa* infections by empirical administration of dual combinations of antibiotics to increase the likelihood of achieving appropriate therapy. The rationale for this is that administration of two agents with different modes of action will increase the chance that the pathogen will be inhibited by at least one of the component drugs. For *P. aeruginosa* infections,
the dual antibiotic combinations usually consist of an anti-
pseudomonal β-lactam with an aminoglycoside or a fluoro-
quinolone (Tamma et al., 2012). Other claimed advantages of 
combination treatments include synergistic inhibition 
and the potential to prevent the selection of resistant 
organisms. One disadvantage is that combination treat-
ments that include an aminoglycoside can result in greater 
nephrotoxicity when compared with the constituent mono-
therapies (Tamma et al., 2012).

Many studies have identified synergistic inhibition of P. 
aeruginosa by diverse dual antibiotic combinations in vitro 
(He et al., 2012; Samonis et al., 2012; Tamma et al., 2012; 
Vidaillac et al., 2012). Crucially, however, studies showing 
clinical evidence of enhanced efficacy of apparently 
synergistic combination treatments over the constituent 
monotherapies are less definitive and have been reviewed 
by Tamma et al. (2012). In fact, in some studies, the 
presence of in vitro synergy did not correlate with clinical 
outcome at all (Chandrasekar et al., 1987; Hilf et al., 1989). 
The fact that in vitro studies cannot take into account 
the variable pharmacokinetics and pharmacodynamics of 
antibiotics, or the presence of an active immune response 
to infection in vivo, could explain the poor correlation 
between in vitro and in vivo outcomes.

Whether or not combination therapy provides real clinical 
benefit for treatment of P. aeruginosa infections remains 
controversial and data from a number of studies are 
conflicting. The topic is comprehensively reviewed by 
Tamma et al. (2012). Clearly, definitive therapy with a 
single antibiotic is the ideal scenario. Evidence of any 
therapeutic benefit from definitive antibiotic combinations 
over definitive monotherapy is unlikely. However, available 
evidence of enhanced efficacy from empirical combination 
therapy over empirical monotherapy is not conclusive 
(Bowers et al., 2013; Vardakas et al., 2013). The situation is 
far more complicated by differences in measured therapeutic 
benefit from monotherapy or combination treatment of 
different P. aeruginosa infections (Tamma et al., 2012). As a 
consequence of this, there have been calls for additional 
research and more definitive studies (Vardakas et al., 2013).

Plainly, there is an urgent clinical need to identify novel 
treatments for MDR P. aeruginosa. It is unlikely that new 
anti-pseudomonal drugs will be developed in the near 
future. Thus, it is necessary to make optimal use of the 
antibiotics that are currently available. Novel combinations 
of antibiotics could still be a potential solution to the 
problem but require initial screening and assessment of 
efficacy in appropriate in vivo infection models rather than 
reliance on in vitro studies to provide the best chance of 
success in patients.

In a recent study conducted in the corresponding author’s 
laboratory, the suitability of a Galleria mellonella larva 
infestation model for the study of the efficacy and pharma-
cokinetics of a range of anti-pseudomonal antibiotics was 
proven (Hill et al., 2013). Employing a characterized MDR 
strain of P. aeruginosa (NCTC 13437), the aim of this study 
was to compare the inhibitory effect of antibiotic combi-
nations in vitro with their efficacy in the G. mellonella 
model in vivo to identify combinations that could provide 
realistic future therapeutic options for treating infections 
with this organism.

METHODS

P. aeruginosa bacteria and growth media. P. aeruginosa NCTC 
13437 was obtained from the National Collection of Type Cultures 
(http://www.hpaclutures.org.uk/collections/nctc.jsp) and was cul-
tured overnight in Mueller–Hinton broth (MHB; Merck) at 37 ºC 
with shaking to prepare inocula for in vitro antibiotic susceptibility testing. NCTC 13437 is MDR and 
possesses two β-lactamases: VEB-1 extended-spectrum β-lactamase and VIM-10 metallo-carbapenemase, that 
render the strain multiply resistant to carbapenems and other β-lactam antibiotics. NCTC 13437 is 
also resistant to quinolones and aminoglycosides by unknown mechanisms (Woodford et al., 2008).

Antibiotics and G. mellonella larvae. All antibiotics [ceftazidime 
(CTX), piperacillin (PIP), meropenem (MER), amikacin (AMK), 
levofloxacin (LVX) and colistin (CST)] and Pseudomonas Isolation Agar (PIA) were purchased from Sigma-Aldrich. All stock solutions 
and substocks were made using sterile deionized water. G. mellonella larvae were purchased from Livefood UK.

Antibiotic susceptibility testing. This was performed exactly as 
previously described (Hill et al., 2013). Briefly, the MIC of each 
antibiotic versus P. aeruginosa NCTC 13437 was determined in 96-
well microplates (Greiner Bio-one) via doubling dilution of each 
antibiotic in MHB and subsequent inoculation with 1.0 × 10⁶ c.f.u. 
ml⁻¹ of P. aeruginosa NCTC 13437. Microplates were incubated at 37 ºC and the MIC was defined as the concentration present in the 
first optically clear well after 24 h. The experiment was performed in 
triplicate.

Time-kill assay of P. aeruginosa viability. Viability of P. 
aeruginosa was determined in 96-well microplates after exposure to 
antibiotics alone or in combination. Aliquots from antibiotic stock 
solutions were added to wells containing MHB, while sterile water 
was added to control wells. Wells were inoculated with 1.0 × 10⁶ c.f.u. 
of P. aeruginosa ml⁻¹ and the plate was incubated at 37 ºC for 24 h. 
Subsequently, the abundance of viable cells in each well was 
determined by serial dilution in MHB and plating on nutrient agar. 
Plates were incubated at 37 ºC for 24 h to permit colonies to form. 
Each treatment was replicated in quadruplicate and a mean value 
calculated. Here, synergy was defined at 24 h as a ≥2-log₁₀ reduction 
in c.f.u. ml⁻¹ by the combination treatment relative to the most 
effective single treatment, so long as the combination reduced the 
starting inoculum by ≥2-log₁₀ c.f.u. ml⁻¹ (White et al., 1996). The 
minimum c.f.u. ml⁻¹ detectable in this assay was 200.

G. mellonella model of P. aeruginosa infection and deter-
mination of G. mellonella haemolymph burden. This was 
performed exactly as previously described (Hill et al., 2013). Unless 
otherwise stated, groups of larvae were infected with an inoculum of 
2.5 × 10⁶ c.f.u. ml⁻¹ of P. aeruginosa NCTC 13437 cells. A single 
treatment of each dual or triple antibiotic combination was 
administered 2 h post-infection (p.i.). For experiments involving 
multiple doses of single antibiotics, or dual and triple antibiotic 
combinations, the second dose was administered 5 h p.i and the 
third dose 8 h p.i. Each experiment used groups containing 15 larvae and 
experiments were repeated twice using larvae from different batches.
The data from these replicate experiments were pooled to give \( n = 30 \). Survival data were plotted using the Kaplan–Meier method and comparisons made between groups using the log rank test. In all comparisons to the negative control it was the uninfected control (rather than the unmanipulated control) that was used. In all tests \( P \leq 0.05 \) was considered significant and Holm’s correction was always applied to account for multiple comparisons (Holm, 1979).

For haemolymph burden, groups of 30 larvae were infected with \( 2.5 \times 10^3 \) or \( 2.5 \times 10^6 \) c.f.u. ml\(^{-1} \) of \( P. \) aeruginosa. As above, single antibiotics or dual and triple combinations were administered at either 2 h p.i. as a single dose or at 2, 5 and 8 h p.i. for multiple dosing. The larvae were incubated in Petri dishes at 37 °C. At 24 h intervals, five larvae were selected at random from each treatment group and tested for haemolymph burden. No discrimination was made between live or dead larvae. Thus, in cases where the tested treatment offered minimal therapeutic benefit to the population, the majority of larvae sampled would be dead. In contrast, if the treatment regimen was successful, the majority of larvae sampled would be live. Selected larvae were anaesthetized and surface disinfected by vortexing in ethanol. The ethanol was poured off and used to homogenize the larva and release the haemolymph. A further 300 µl of sterile PBS was then added to each tube and vortexed. Twenty microlitres of the larval homogenate was serially diluted in 180 µl of MHB in a 96-well microplate (Greiner Bio-one). This process was repeated for each of the five larvae sampled per treatment group every 24 h. The dilution series was plated on PIA plates and incubated at 37 °C for 24 h. Colonies were counted after 24 h and the data expressed as viable c.f.u. ml\(^{-1} \). Using this method the detection limit was 100 c.f.u. ml\(^{-1} \) of larval homogenate.

**RESULTS**

**Dual and triple combinations of antibiotics display synergistic inhibition of MDR \( P. \) aeruginosa in vitro**

The MICs of the antibiotics employed in this study (CTX, PIP, MER, CST, LVX and AMK) versus \( P. \) aeruginosa NCTC 13437 are shown in Table 1. These MIC values verify those already published for this strain and further confirm that the strain has an MDR phenotype with high MIC values for all the drugs tested compared with a drug-sensitive strain (with the exception of colistin) (Woodford et al., 2008; Hill et al., 2013). Subsequently, the inhibitory effect of a 24 h exposure to the individual drugs alone at MIC\(_{0.5}\) and the 15 possible dual combinations of the six antibiotics tested at MIC\(_{0.5}\) was measured (Table 2). With the exception of CST, exposure to the MIC\(_{0.5}\) of each antibiotic alone had no inhibitory effect on growth compared with the untreated control. Exposure to 1 mg l\(^{-1} \) of CST did result in \( >2 \log_{10} \) c.f.u. ml\(^{-1} \) reduction over 24 h but did not reduce the cell numbers below the level of the initial inoculum. Out of the 15 dual antibiotic combinations, 10 reduced cell numbers below the detectable limit of the assay (2.3 log\(_{10} \) c.f.u. ml\(^{-1} \)) compared with the untreated control and could be classed as synergistic (White et al., 1996). Notably, of the 10 synergistic dual combinations, eight had one component \( \beta \)-lactam antibiotic and one consisted of two \( \beta \)-lactams (MER and PIP). The remaining synergistic combination consisted of CST with LVX. These 10 synergistic dual combinations were then tested again at MIC\(_{0.25}\) and no significant inhibitory effects were detected for any of the combinations at these lower concentrations (data not shown).

The effect of a 24 h exposure to the 20 possible triple antibiotic combinations at MIC\(_{0.25}\) on \( P. \) aeruginosa NCTC 13437 is shown in Table 3. Only four out of 20 triple combinations showed synergistic inhibition and resulted in a reduction in cell numbers below the detectable limit of the assay (2.3 log\(_{10} \) c.f.u. ml\(^{-1} \)) compared with the untreated control. All four of these synergistic triple combinations included two \( \beta \)-lactam antibiotics and three of the combinations also included the aminoglycoside AMK. At MIC\(_{0.125}\) no inhibition was detected compared with the untreated control (data not shown).

**Dual and triple combinations of antibiotics show enhanced efficacy compared with constituent monotherapies versus MDR \( P. \) aeruginosa infection in vivo**

The efficacy of all the antibiotic combinations was measured in vivo using \( G. \) mellonella larvae infected with \( P. \) aeruginosa NCTC 13437 (Table 4). This experiment also allowed for comparison of the merit of screening for novel treatments in vitro versus an actual infection in vivo.

For each antibiotic combination, a dose of each constituent antibiotic was selected that alone provided minimal therapeutic benefit to larvae infected with \( P. \) aeruginosa NCTC 13437 (Hill et al., 2013). This approach to dosing allowed for easy detection of any enhanced efficacy that arose upon administration of antibiotic combinations. Of the 15 possible dual combination treatments, only three showed significantly enhanced 96 h survival of infected \( G. \) mellonella at the lowest inoculum tested (2.5 \( \times 10^3 \) c.f.u. ml\(^{-1} \)). The best of these was the combination of two \( \beta \)-lactams (CTX+PIP), followed by PIP+AMK and PIP+LVX.

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**Table 1. MIC of antibiotics used in this study versus \( P. \) aeruginosa NCTC 13437**

The MIC (mg l\(^{-1} \)) of each antibiotic was determined in MHB after 24 h incubation at 37 °C. The experiment was performed in triplicate.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>( P. ) aeruginosa NCTC 13437</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTX</td>
<td>512</td>
</tr>
<tr>
<td>PIP</td>
<td>128</td>
</tr>
<tr>
<td>MER</td>
<td>64</td>
</tr>
<tr>
<td>CST</td>
<td>2–4</td>
</tr>
<tr>
<td>LVX</td>
<td>64</td>
</tr>
<tr>
<td>AMK</td>
<td>16</td>
</tr>
</tbody>
</table>
Table 2. The effect of dual combinations of antibiotics at \text{MIC}_{0.5} on the viability of \( P. \) aeruginosa NCTC 13437

Viability was determined in 96-well microplates after 24 h exposure to the antibiotics in MHB at 37 °C. Shaded combinations are those that displayed synergistic killing. Data shown are the mean and standard deviation from quadruple experiments.

<table>
<thead>
<tr>
<th>Antibiotic(s)</th>
<th>Concentration (mg l(^{-1}))</th>
<th>Inoculum</th>
<th>Log c.f.u. ml(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single treatment at \text{MIC}_{0.5}</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTX</td>
<td>256</td>
<td></td>
<td>9.22 ( \pm ) 0.02</td>
</tr>
<tr>
<td>PIP</td>
<td>64</td>
<td></td>
<td>9.17 ( \pm ) 0.14</td>
</tr>
<tr>
<td>MER</td>
<td>32</td>
<td>6.09 ( \pm ) 0.015</td>
<td>9.32 ( \pm ) 0.069</td>
</tr>
<tr>
<td>CST</td>
<td>1</td>
<td></td>
<td>9.30 ( \pm ) 0.10</td>
</tr>
<tr>
<td>LVX</td>
<td>32</td>
<td></td>
<td>6.85 ( \pm ) 1.14</td>
</tr>
<tr>
<td>AMK</td>
<td>8</td>
<td></td>
<td>8.97 ( \pm ) 0.93</td>
</tr>
<tr>
<td>Double combinations at \text{MIC}_{0.5}</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTX+MER</td>
<td>256 + 32</td>
<td></td>
<td>( \leq ) 2.30</td>
</tr>
<tr>
<td>CTX+PIP</td>
<td>256 + 32</td>
<td></td>
<td>9.47 ( \pm ) 0.09</td>
</tr>
<tr>
<td>CTX+AMK</td>
<td>256 + 8</td>
<td></td>
<td>9.18 ( \pm ) 0.06</td>
</tr>
<tr>
<td>CTX+LVX</td>
<td>256 + 32</td>
<td></td>
<td>( \leq ) 2.30</td>
</tr>
<tr>
<td>CTX+CST</td>
<td>1 + 8</td>
<td>6.09 ( \pm ) 0.015</td>
<td>9.32 ( \pm ) 0.029</td>
</tr>
<tr>
<td>MER+PIP</td>
<td>32 + 64</td>
<td></td>
<td>( \leq ) 2.30</td>
</tr>
<tr>
<td>MER+CST</td>
<td>32 + 1</td>
<td></td>
<td>( \leq ) 2.30</td>
</tr>
<tr>
<td>MER+AMK</td>
<td>32 + 8</td>
<td></td>
<td>( \leq ) 2.30</td>
</tr>
<tr>
<td>MER+LVX</td>
<td>32 + 32</td>
<td></td>
<td>( \leq ) 2.30</td>
</tr>
<tr>
<td>PIP+CST</td>
<td>64 + 1</td>
<td></td>
<td>( \leq ) 2.30</td>
</tr>
<tr>
<td>PIP+AMK</td>
<td>64 + 8</td>
<td></td>
<td>( \leq ) 2.30</td>
</tr>
<tr>
<td>PIP+LVX</td>
<td>64 + 32</td>
<td></td>
<td>( \leq ) 2.30</td>
</tr>
<tr>
<td>CST+LVX</td>
<td>1 + 32</td>
<td></td>
<td>( \leq ) 2.30</td>
</tr>
<tr>
<td>CST+AMK</td>
<td>1 + 8</td>
<td>6.02 ( \pm ) 0.71</td>
<td>3.30</td>
</tr>
<tr>
<td>AMK+LVX</td>
<td>8 + 32</td>
<td>5.84 ( \pm ) 0.64</td>
<td>3.48</td>
</tr>
</tbody>
</table>

Following this, the 20 possible triple antibiotic combinations were tested in the same way. Of these 20 combinations, 15 showed significantly enhanced 96 h survival of larvae infected with the lowest inoculum \((2.5 \times 10^3 \text{ c.f.u. ml}^{-1})\). All of these 15 combinations included at least one \( \beta \)-lactam. To further discriminate the optimal antibiotic combinations, the experiment was repeated versus larvae infected with an intermediate inoculum \((2.5 \times 10^4 \text{ c.f.u. ml}^{-1})\). This eliminated eight of the triple combinations with the seven remaining still showing significantly enhanced survival. The optimal triple combination was MER+PIP+AMK followed by CTX+PIP+AMK. Notably, both of these combinations include two \( \beta \)-lactams with an aminoglycoside. A single dose of all of these combinations had no therapeutic benefit on larvae inoculated with a high number of bacteria \((2.5 \times 10^6 \text{ c.f.u. ml}^{-1})\).

The therapeutic benefit arising from treatment with antibiotic combination treatments correlates with reduced larval burden of infecting MDR \( P. \) aeruginosa

The effect of the most potent antibiotic combination treatments identified above on the larval burden of \( P. \) aeruginosa was compared with the effect of their constituent mono- or dual therapies. The effect of the dual combination of CTX+PIP is shown in Fig. 1. Notably, exposure of infected larvae to a single dose of 100 mg kg\(^{-1}\) CTX+70 mg kg\(^{-1}\) PIP resulted in significantly enhanced survival \((P \leq 0.05)\) compared with monotherapy with either 100 mg kg\(^{-1}\) CTX or 100 mg kg\(^{-1}\) PIP (Fig. 1a). Measurement of larval burden of \( P. \) aeruginosa showed that treatment with either 100 mg kg\(^{-1}\) CTX or 70 mg kg\(^{-1}\) PIP alone had no inhibitory effect as there was rapid proliferation of \( P. \) aeruginosa within the larvae over a period of 24 h (Fig. 1b) and the numbers of \( P. \) aeruginosa recovered were similar to those from infected larvae treated with PBS alone (Hill et al., 2013). In contrast, a single dose of the combination of CTX+PIP prevented the rapid growth of \( P. \) aeruginosa over 24 h and resulted in a significant reduction in the number of viable bacteria present within the larvae over the entire 96 h duration of the experiment (Fig. 1b).

The effect of treatment with the optimal triple combination of MER+PIP+AMK on larval burden of \( P. \) aeruginosa was also examined (Fig. 2). A single dose of 2 mg kg\(^{-1}\) MER+70 mg kg\(^{-1}\) PIP+50 mg kg\(^{-1}\) AMK resulted in greater than 80% survival of infected larvae compared with the PBS-treated control. Treatment with the triple combination also resulted in significantly greater survival than any of the possible constituent dual therapies \((P \leq 0.05)\) (Fig. 2a). Confirming the data in Table 4, a single dose of the dual combination of 70 mg kg\(^{-1}\) PIP+50 mg kg\(^{-1}\) AMK did result in significantly
enhanced survival compared with the PBS-treated control and the other two constituent dual combinations of AMK+MER and PIP+MER. However, the therapeutic benefit obtained from the triple therapy was significantly greater ($P \leq 0.05$) than that provided by PIP+AMK.

The dual combinations of AMK+MER and PIP+MER offered no therapeutic benefit over 96 h and this was reflected in rapid proliferation of *P. aeruginosa* within the larvae during the first 24 h post-treatment (Fig. 2b; only data for AMK+MER are shown for clarity). Treatment with either the triple combination or the constituent double combination PIP+AMK resulted in significant reductions in the number of viable *P. aeruginosa* recovered from the larvae compared with treatment with either AMK+MER or PIP+MER ($P \leq 0.05$). Reflecting the optimal larval survival seen after treatment with the triple combination compared with the double combination of PIP+AMK, there was an indication that the median numbers of bacteria recovered at 72 and 96 h from the infected larvae treated with the triple combination were approximately 1 log$_{10}$ c.f.u. ml$^{-1}$ less than larvae treated with PIP+AMK, although this difference was not found to be significant ($P \geq 0.05$) (Fig. 2b).

### Multiple dosing with the triple combination of MER+PIP+AMK is efficacious versus an infection with high numbers of MDR *P. aeruginosa* in vivo

The effect of multiple doses of the optimal triple combination (MER+PIP+AMK) on larvae infected with a high inoculum of MDR *P. aeruginosa* ($2.5 \times 10^6$ c.f.u. ml$^{-1}$) is shown in Fig. 3. Firstly, three doses of either MER, PIP or AMK alone had no significant therapeutic benefit on infected larvae over 96 h compared with treatment with PBS alone (Fig. 3a). Three doses of the constituent dual therapies of the triple combination (PIP+AMK, AMK+MER and PIP+MER) all resulted in significantly enhanced survival compared with infected larvae treated with PBS alone ($P \leq 0.05$). However, the therapeutic benefit conferred by three doses of the triple combination was significantly greater than any of the dual antibiotic combinations ($P \leq 0.05$) (Fig. 3b).

### Table 3. The effect of triple combinations of antibiotics at MIC$_{0.25}$ on the viability of *P. aeruginosa* NCTC 13437

Viability was determined in 96-well microplates after 24 h exposure to the antibiotics in MHB at 37 °C. Shaded combinations are those that displayed synergistic killing. Data shown are the mean and standard deviation from quadruple experiments.
Table 4. Efficacy of double and triple antibiotic combination treatments versus G. mellonella larvae infected with P. aeruginosa NCTC 13437

One dose of each treatment was administered 2 h p.i. and survival measured 96 h p.i. * Indicates significantly enhanced survival compared to PBS treatment (P<0.05, log rank test with Holm correction for multiple comparisons); n=30 (pooled from duplicate experiments). –, Not tested. Shading indicates the most potent combinations.

<table>
<thead>
<tr>
<th>Therapy</th>
<th>Antibiotic(s)</th>
<th>Dose (mg kg(^{-1}))</th>
<th>Low inoculum (%) survival (in vivo) 96 h p.i.</th>
<th>Intermediate inoculum (%) survival (in vivo) 96 h p.i.</th>
<th>High inoculum (%) survival (in vivo) 96 h p.i.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single treatment</td>
<td>CTX</td>
<td>100</td>
<td>0</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>PIP</td>
<td>70</td>
<td>0</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>MER</td>
<td>2</td>
<td>0</td>
<td>–</td>
<td>–</td>
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<td></td>
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<td></td>
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<tr>
<td>Double combinations</td>
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<td>7</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>CTX + PIP</td>
<td>100 + 70</td>
<td>80*</td>
<td>–</td>
<td>–</td>
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<tr>
<td></td>
<td>CTX + AMK</td>
<td>100 + 50</td>
<td>13*</td>
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<td>100 + 100</td>
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<td>–</td>
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<tr>
<td></td>
<td>CTX + CST</td>
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<td>–</td>
<td>–</td>
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<td>MER + PIP</td>
<td>2 + 70</td>
<td>0</td>
<td>–</td>
<td>–</td>
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<tr>
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combination did not result in enhanced survival of larvae infected with a high inoculum over the duration of the experiment (Fig. 3c). Both two and three doses of the triple combination did confer significantly enhanced survival compared with PBS-treated larvae (P<0.05) but optimal therapeutic benefit was conferred by three doses (Fig. 3c). The ineffective single dose of the triple combination did not prevent rapid proliferation of P. aeruginosa within the larvae over the first 48 h of the experiment (Fig. 3d). In contrast, both the two and three dose regimens of the triple combination prevented this proliferation; in both cases the median value of recovered bacteria was significantly reduced when compared with bacterial numbers recovered from larvae exposed to a single dose. Notably, despite the three dose...
treatment offering significantly enhanced survival of infected larvae compared with just two doses (Fig. 3c), there was no significant difference in the number of \( P. \text{aeruginosa} \) recovered between the two treatment regimens (\( P \geq 0.05 \)) (Fig. 3d).

**DISCUSSION**

\( G. \text{mellonella} \) larvae were shown recently to represent a highly effective model to test the efficacy of antibiotic treatments for \( P. \text{aeruginosa} \) infections *in vivo*. The efficacy of a range of antibiotics on larvae infected with an antibiotic-sensitive isolate or an MDR strain closely correlated with the known drug susceptibilities of the two strains *in vitro* and in patients (Hill *et al.*, 2013). Hence, the \( G. \text{mellonella} \) larval model offers real advantages for screening the effectiveness of novel anti-pseudomonal treatments due to its rapidity, low cost and lack of ethical concerns in comparison to the use of mammals.

In the work reported here, \( G. \text{mellonella} \) was used to screen the efficacy of multiple antibiotic combinations *in vivo*...
versus *P. aeruginosa* infection and compared with the inhibitory effect of the same combinations in an *in vitro* 24 h kill assay. A number of dual and triple combinations of anti-pseudomonal antibiotics demonstrated synergistic inhibition of MDR *P. aeruginosa* in vitro. This finding is not new and confirms a plethora of data within the literature which has been reviewed by Tamma *et al.* (2012). However, comparison of the efficacy of the same combinations *in vivo* using *G. mellonella* larvae infected with MDR *P. aeruginosa* revealed little correlation with the inhibitory effects measured *in vitro*. For example, the dual combination of CTX + PIP significantly enhanced survival compared with the best dual combination of PIP + AMK (\(P<0.05\), log rank test with Holm correction for multiple comparisons); \(n=30\) (pooled from duplicate experiments).
dual combinations that showed enhanced efficacy for effective antibiotic combinations calls into question the utility of conducting initial screening clinical studies in patients (see Introduction) and further two included the combination of a but displayed no synergistic inhibition under the conditions of the in vitro studies that compared results generated in vivo for efficacious antibiotic combination treatments et al. 1985; Al-Hasan #, treatment with one dose resulted in significantly greater survival than with PBS; ~, treatment with three doses resulted in significantly greater survival than with one dose; ~, treatment with two doses resulted in significantly greater survival than with one dose; *, treatment with three doses resulted in significantly greater survival than with one dose; #, treatment with two doses resulted in significantly greater survival than with one dose; #, treatment with one dose resulted in significantly greater survival than with PBS; #, treatment with two doses resulted in significantly greater survival than with one dose; *, significantly enhanced survival compared with PBS treatment; #, treatment PIP + AMK + MER induced significantly greater survival than AMK + MER, PIP + MER or PIP + AMK. (c) Effect of one (2 h p.i), two (2 and 5 h p.i) or three (2, 5 and 8h p.i) doses of the triple combination PIP + AMK + MER, *© (d) Effect of one, two or three doses of the triple combination of PIP + MER, PIP or AMK) or the triple combination (PIP + AMK + MER). *, significantly enhanced survival compared with PBS treatment; #, treatment PIP + AMK + MER induced significantly greater survival than AMK + MER, PIP + MER or PIP + AMK. (c) Effect of one (2 h p.i), two (2 and 5 h p.i) or three (2, 5 and 8h p.i) doses of the triple combination PIP + AMK + MER, *© using G. mellonella, available evidence suggests that the data generated are more likely to be predictive of efficacy in patients than in vitro assays.

The most potent dual and triple antibiotic combinations that were identified using the G. mellonella model consisted of CTX + PIP and MER + PIP + AMK, respectively. Notably, out of seven triple antibiotic combination treatments shown to provide enhanced therapeutic benefit in vivo, only two were shown to be synergistic in vitro. Importantly, these findings imply that if screening of novel antimicrobials or combinations of antibiotics is performed initially in vitro, then treatments that are shown to be ineffective under these conditions would be discarded despite still having the

in vivo but displayed no synergistic inhibition under the conditions of the in vitro assay. This supports previous studies that compared results generated in vitro with clinical studies in patients (see Introduction) and further calls into question the utility of conducting initial screening for effective antibiotic combinations in vitro. Of the three dual combinations that showed enhanced efficacy in vivo, two included the combination of a β-lactam with either an aminoglycoside or a fluoroquinolone; such antibiotic combinations have been shown in some studies to offer enhanced outcomes compared with monotherapy in patients suffering from Gram-negative infections (Bodey et al., 1985; Al-Hasan et al., 2009). Therefore, by screening for efficacious antibiotic combination treatments in vivo

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potential to be effective in vivo. Similarly, as this study shows, a large number of antibiotic combinations were identified as being potently synergistic in vitro but offered no enhanced therapeutic benefit over monotherapies when tested in vivo. Thus, the case for utilizing the G. mellonella model for initial screening of the effectiveness of novel antimicrobials, or combinations of antibiotics, is convincing.

The demonstration that the combination of CTX + PIP offered enhanced therapy in vivo is notable. Furthermore, out of seven triple antibiotic combinations that displayed enhanced therapeutic benefit in vivo, one consisted of three, and four included at least two β-lactams. Most attempts to identify synergistic combinations of antibiotics combine drugs with different inhibitory modes of action, with the obvious rationale that the inhibition of multiple bacterial targets is likely to lead to additive or synergistic inhibition that would be more potent than hitting a single essential target. Consequently, assessing the effectiveness of combinations of β-lactams that purportedly inhibit the same molecular targets is not intuitive; thus it is unlikely that they would be included in any screens. Furthermore, even if combinations of β-lactams were screened in vitro, as this work has shown, most would not be identified as synergistic and subsequently would never be evaluated in vivo. The consequence of this is that there could be a range of effective antibiotic combinations yet to be identified that could have the potential for clinical application.

Evidence of the effectiveness of dual β-lactam antibiotic combinations providing synergistic inhibition of P. aeruginosa in vitro is extensive (Scribner et al., 1982; Dales et al., 2009). Oie et al. (2003) identified that the dual combination of PIP and ceftazidime significantly inhibited two out of seven strains of MDR P. aeruginosa. In the same study, triple combinations of β-lactams, including PIP, MER and ceftazidime, or PIP, ceftazidime and aztreonam, had significant activity against three and five of the seven strains, respectively. Supporting our findings, the authors reported that the triple combination of MER, PIP and AMK (the most potent triple combination that was identified using the G. mellonella infection model) inhibited three out of the seven MDR P. aeruginosa strains tested in vitro.

Notably, there are few published studies on the efficacy of β-lactam combinations in vivo. One study compared the efficacy of cefoperazone with PIP, ceftazidime with PIP, and imipenem alone on febrile granulocytopenic patients and concluded that the dual antibiotic combinations were as effective as the monotherapy but offered no enhanced therapeutic benefit (Winston et al., 1991).

In summary, using G. mellonella, we have presented evidence that a dual combination of CTX with PIP, and triple combinations of antibiotics that include at least two different β-lactams, result in enhanced therapeutic benefit compared with monotherapies versus MDR P. aeruginosa infection in vivo. Furthermore, this study has emphasized the inadequacy of screening for novel treatments in vitro and highlighted the potential for carrying out these screens in vivo using G. mellonella larvae. In future, we plan to assess the efficacy of the novel antibiotic combinations that were identified in G. mellonella in suitable mammalian models to further develop their potential for clinical application.

ACKNOWLEDGEMENTS

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REFERENCES


