Enhanced efficacy of putative efflux-pump inhibitor/antibiotic combination treatments versus MDR strains of *Pseudomonas aeruginosa* in a *Galleria mellonella in vivo* infection model.

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Running title: Efflux-pump inhibitor/antibiotic combination therapy

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Synopsis

Objectives - To compare the antibiotic susceptibility of *Pseudomonas aeruginosa* strains with increased efflux-pump expression *in vitro* and *in vivo* and to use these same strains to evaluate the efficacy of combinations of antibiotics with putative efflux-pump inhibitors *in vivo*.

Methods – A collection of *P. aeruginosa* strains that over-express three efflux-pumps (MexAB-OprM, MexCD-OprJ, MexEF-OprN), in addition to a strain with all three Mex pumps deleted, were used. The virulence of these strains and their antibiotic susceptibility was measured *in vivo* using a *Galleria mellonella* larval infection model. The inhibitory effect of combinations of putative efflux-pump inhibitors (trimethoprim and sertraline) with antibiotics on the strain over-expressing MexAB-OprM was also measured *in vitro* and compared with their efficacy *in vivo* in terms of larval survival and bacterial burden.

Results – Increased expression of the individual efflux-pumps, or deletion of all three, had no significant effect on the virulence of *P. aeruginosa* *in vivo*. Expression levels of the efflux-pumps clearly influenced antibiotic efficacy *in vivo*. The efficacy of levofloxacin, piperacillin and meropenem versus larvae infected with the efflux-pump mutants reflected susceptibility to the same drugs *in vitro*. Treatment of *G. mellonella* larvae infected with a strain that over-expressed MexAB-OprM with a combination of putative efflux-pump inhibitors and levofloxacin resulted in enhanced therapeutic benefit compared to the constituent monotherapies.

Conclusions - This study has demonstrated the utility of using *G. mellonella* to screen for novel therapeutic options for MDR *P. aeruginosa* and has shown that antibiotic/efflux-pump inhibitor combinations should be further investigated for clinical application.
Introduction

*Pseudomonas aeruginosa* is a ubiquitous, opportunistic pathogen prominent in hospitalised, immunocompromised patients where it targets the urinary and respiratory tracts, kidneys and bloodstream principally via invasive fomites such as catheters and mechanical ventilators. Treatment of *P. aeruginosa* infections can be difficult due to its high intrinsic resistance, which in turn has been amplified by the rapid spread of resistance determinants and arrival of the MDR phenotype where isolates are resistant to three or more different classes of antibiotics.

The over-expression of membrane-associated efflux-pumps that contribute to reduced efficacy of a number of important classes of antibiotics used against *P. aeruginosa* is an important contributor to resistance. The most significant family of efflux-pumps that mediate antibiotic resistance in *P. aeruginosa* are those belonging to the resistance-nodulation-division (RND). Four of the twelve member RND family are strongly associated with antibiotic efflux: MexAB-OprM, MexXY-OprM, MexCD-OprJ and MexEF-OprN. These four pumps transport an overlapping range of antibiotic substrates, in particular the fluoroquinolones. However, MexAB-OprM is the least discriminatory, with a broad substrate range allowing extrusion of almost all classes of antibiotic, and is the most important efflux-pump mediating antibiotic resistance because it is constitutively expressed and responsible for much of the intrinsic resistance of the organism. Knockout of *mexAB-oprM* results in hyper-susceptibility to antibiotics. In contrast, expression of MexCD-OprJ and MexEF-OprN requires exposure to a broad range of compounds or environmental stimuli. MexCD-OprJ’s substrate range mirrors that of MexAB-OprM while MexEF-OprN appears the least versatile. Knockout of either of these two pumps does not affect antibiotic susceptibility. Mutations in efflux-pump regulatory genes can result in over-expression of any of these pumps and confer a multi-drug resistant phenotype. Importantly, these mutations are commonly identified in clinical isolates, for example, mutations in *nalB*, *ntxB* and *nfxC* result in over-expression of MexAB-OprM, MexCD-OprJ and MexEF-OprN respectively.

The role of efflux-pumps in mediating resistance to antibiotics has led to efforts to inhibit their activity as a strategy to reduce the level of antibiotic resistance. Identifying combination treatments of efflux-pump inhibitors (EPIs) with these antibiotics could result in the restoration of antibiotic activity.

In two independent studies, evidence was presented that the antibiotic trimethoprim and the selective serotonin reuptake inhibitor sertraline were synergistic in combination with a range of antibiotics versus Gram-negative bacteria *in vitro*. Both drugs demonstrated EPI-like activity by inhibiting the efflux of dyes.
from these bacteria. Using a collection of characterised *P. aeruginosa* strains that over-express three RND efflux pumps (MexAB-OprM, MexCD-OprJ, MexEF-OprN), the aim of this study was to assess the efficacy of combinations of antibiotics with sertraline and trimethoprim using a *Galleria mellonella* infection model *in vivo* to determine if these combinations could represent a future treatment option for infections with *P. aeruginosa* strains that display an efflux-pump mediated MDR phenotype.

**Materials and Methods**

*Bacteria and growth media.* The strains of *P. aeruginosa* used in this study are shown in Table 1. Strain PAO1 and the efflux pump mutants were a kind gift of Dr. Olga Lomovskaya, Rempex Pharmaceuticals, USA. *P. aeruginosa* NCTC13437 was obtained from the National Collection of Type Cultures (NCTC) (http://www.phe-culturecollections.org.uk/collections/nctc.jsp). All strains were cultured overnight in Mueller-Hinton Broth (MHB; Merck, Darmstadt, Germany) at 37°C with shaking to prepare inocula for drug susceptibility testing *in vitro* and efficacy testing *in vivo*.

*Antibiotics and G. mellonella larvae.* All antibiotics: ceftazidime (CFD), piperacillin (PIP), meropenem (MER), amikacin (AMK), levofloxacin (LVX) and colistin (CST) and Pseudomonas Isolation Agar (PIA) were purchased from Sigma-Aldrich Ltd (Dorset, UK). Stock solutions and sub-stocks were made using sterile deionised water. Putative efflux-pump inhibitors sertraline HCl (SER), trimethoprim (TMP) and PAβN were purchased from Sigma-Aldrich Ltd. Stock solutions of sertraline and trimethoprim were made in 50% and 75% (v/v) DMSO respectively. PAβN was made in sterile deionised water. Sub-stocks of all drugs for use in experiments were made in sterile deionised water. *G. mellonella* larvae were purchased from UK Waxworms Ltd (Sheffield, UK).

*Drug susceptibility testing.* This was performed exactly as previously described. Briefly, the MIC of each antibiotic and the putative EPIs versus *P. aeruginosa* strains was determined in 96-well microplates (Greiner Bio-one Ltd, Stonehouse, UK) via doubling dilution of each drug in MHB and subsequent inoculation with 1.0x10⁶ cfu/mL of *P. aeruginosa*. The MIC value of combinations of antibiotics with EPIs was carried out exactly as above using 96-well microplate assays prepared via doubling dilution of each antibiotic in MHB followed by addition of a single concentration of each EPI. All three putative EPIs (trimethoprim, sertraline and PAβN) were tested in combination with levofloxacin, piperacillin and
meropenem. Each EPI was screened in combination with the antibiotics at concentrations over three orders of magnitude that were lower than the MIC values for each EPI. Microplates were incubated at 37°C and the MIC was defined as the concentration present in the first optically clear well after 24 h. Each experiment was performed in triplicate. Fractional inhibitory concentration index (FICI) values were calculated for each combination tested, and synergy was defined as FICI ≤0.5.

G. mellonella model of P. aeruginosa infection and determination of G. mellonella haemolymph burden.

This was performed exactly as previously described. Unless otherwise stated, groups of larvae were infected with an inoculum of 2.5x10^3 cfu/mL of P. aeruginosa cells. For experiments involving a single dose of levofloxacin (0.2, 1 or 5 mg/kg), piperacillin (10, 25 or 100 mg/kg) or meropenem (0.1, 0.25 or 0.5 mg/kg), the antibiotics were administered 2 h post-infection (p.i). For experiments involving triple doses of either single drugs or dual drug combinations, 1 and 5 mg/kg levofloxacin were used for PAM1020 and PAM1032, respectively. A dose of 100 mg/kg of either trimethoprim or sertraline was used in combination with levofloxacin as pilot studies using both PAM1020 and PAM 1032 revealed this dose resulted in optimal results (data not shown). Triple doses were administered 2, 4 and 6 h p.i. Each experiment used groups containing 15 larvae and experiments were performed 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 ≤0.05 was considered significant and Holm’s correction was always applied to account for multiple comparisons.

For haemolymph burden, groups of 30 larvae were infected with 2.5x10^3 cfu/mL of P. aeruginosa. As above, single drug or dual combinations were administered at either 2 h p.i. as a single dose or at 2, 4 and 6 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 exactly as previously described.

Results

Expression levels of efflux-pumps do not influence virulence of P. aeruginosa in G. mellonella larvae.

Groups of larvae were infected with each of the four mutant strains of P. aeruginosa with altered efflux...
pump expression and the isogenic parent strain (Supplementary Figure 1). With all strains tested, the heat-killed \(P.\ aeruginosa\) inoculum (inoculation dose of \(2.5 \times 10^8\) cfu/mL) had no significant detrimental effect on larval survival \((P>0.05)\) indicating that infection with live bacteria is required to cause larval death.

Inoculation with 25 cfu/larva of each strain resulted in 100% lethality after 24 h compared to controls sham-infected with PBS. No difference in virulence between the strains was discernible. Reducing the inoculum size 10-fold to 2.5 cfu/larva allowed better discrimination of any differences in virulence between the strains. Despite some apparent differences in larval survival 48 h post-infection \((p.i)\), after 96 h there was no significant difference \((P \geq 0.05)\) in survival of larvae infected with any of the strains tested. In conclusion, alteration of efflux-pump expression had no significant effect on the virulence of \(P.\ aeruginosa\) in \(G.\ mellonella\) larvae.

**Antibiotic susceptibility of \(P.\ aeruginosa\) strains with altered expression levels of efflux-pumps is affected in vitro and this correlates with the efficacy of these antibiotics versus \(G.\ mellonella\) larvae infected with the same strains.** In vitro susceptibility of the parent strain and four efflux-pump mutants to a range of anti-Pseudomonal antibiotics is shown in Table 2. Over-expression of mexAB-oprM (PAM1032) conferred resistance to ceftazidime, piperacillin, meropenem and levofloxacin compared to the isogenic parent (PAM1020). Over-expression of mexCD-oprJ (PAM1033) and mexEF-oprN (PAM1034) conferred significant resistance to levofloxacin only. The mutant with all three efflux pumps deleted (PAM1626) displayed susceptibility to ceftazidime, piperacillin, meropenem and levofloxacin compared to the parent (PAM1020). Together, these results are consistent with many previous studies on the substrate specificity of these efflux-pumps.  

To study the effect of altered efflux pump expression on *in vivo* antibiotic efficacy infected larvae were treated with levofloxacin, piperacillin and meropenem as these three drugs had the largest differences in susceptibility between the mutant and parent strains *in vitro* (see Table 2). Larvae were infected with each strain of \(P.\ aeruginosa\) and the effect of a single, increasing dose of each antibiotic on survival measured (Figure 1). Treatment with 0.2 mg/kg of levofloxacin conferred no therapeutic benefit on larvae infected with any of the \(P.\ aeruginosa\) strains tested. Notably, a single dose of 1 mg/kg levofloxacin only induced therapeutic benefit on larvae infected with the mutant with all three efflux pumps deleted (PAM1626). Increasing the dose to 5 mg/kg levofloxacin resulted in almost complete survival of larvae infected with the
tripl
e deletion mutant but also the parent strain (PAM1020). Notably, this highest dose of levofloxacin
offered no therapeutic benefit to larvae infected with the three mutant strains that over-expressed the three
efflux pumps (PAM1032, PAM1033 and PAM1034). A similar trend of antibiotic efficacy, dependent on the
level of efflux-pump expression, was also observed when larvae infected with each strain were treated with
increasing doses of piperacillin. Similar to treatment with levofloxacin and piperacillin, treatment with
meropenem was most effective on larvae infected with the mutant with all three efflux pumps deleted
(PAM1626). Treatment with 0.25 or 0.5 mg/kg meropenem proved to be efficacious to larvae infected with
the parent strain (PAM1020) and the strains over-expressing mexCD-oprJ (PAM1033) or mexEF-oprN
(PAM1034). Notably, over-expression of mexAB-oprM (PAM1032) abolished this efficacy.

In conclusion, the expression level of efflux-pumps clearly influences antibiotic efficacy in vivo.
Furthermore, the in vivo efficacy of levofloxacin, piperacillin and meropenem versus larvae infected with P.
aeruginosa strains displaying altered expression of efflux-pumps reflected exactly the degree of
susceptibility to the same drugs that was measured in vitro.

Treatment of G. mellonella larvae infected with MDR strains of P. aeruginosa with a combination of putative
efflux-pump inhibitors and levofloxacin results in enhanced therapeutic benefit compared to the constituent
monotherapies. The antibiotic trimethoprim\(^9\) and the selective serotonin reuptake inhibitor sertraline\(^10\)
represent two clinically-approved drugs that have also been shown to be putative inhibitors of Gram-
negative efflux-pumps. The MICs of these two drugs and the established EPI PAβN\(^17\) are shown in
Supplementary Table 1. Only the mutant with all three efflux-pumps deleted (PAM1626) displayed any
substantial susceptibility, and the three strains over-expressing individual efflux pumps had a higher MIC
for trimethoprim than the parent, implying that all three putative EPIs may be substrates of these Mex
efflux-pumps. MIC and FICI values of the three EPIs in combination with levofloxacin, piperacillin and
meropenem are shown in Table 3. Combination MICs were determined for the parent strain (PAM1020),
the triple efflux-pump deletion (PAM1626) and the mutant over-expressing mexAB-oprM (PAM1032).
PAM1032 was included over the mutants over-expressing mexCD-oprJ and mexEF-oprN because this
strain was the most resistant to levofloxacin, piperacillin and meropenem treatment in vivo and MexAB-
oprM is responsible for intrinsic resistance of P. aeruginosa to many antibiotics. Combination of
trimethoprim, sertraline and PAβN with levofloxacin each resulted in a reduction in the MIC of levofloxacin
versus the parent strain (PAM1020). The calculated FICI values for each of these combinations indicated synergistic inhibition (≤0.5). Synergy was also observed against the parent strain for combinations of piperacillin with trimethoprim and PAβN, and meropenem with sertraline. None of the putative EPI/antibiotic combinations showed significant synergy versus the strain with all three efflux-pumps deleted (PAM1626).

In contrast to the parent strain (PAM1020), trimethoprim and sertraline did not act in synergy with the antibiotics versus the mutant over-expressing mexAB-oprM (PAM1032). Only the established efflux-pump inhibitor PAβN was synergistic in combination with all three antibiotics against this mutant.

Antibiotic and EPI combinations were then investigated in the G. mellonella infection model for in vivo efficacy. Irrespective of the P. aeruginosa strain investigated, treatment of infected larvae with PAβN (at 100mg/kg) in combination with levofloxacin or piperacillin (both at strain specific doses) did not confer any therapeutic benefit over antibiotic treatment alone (P≥0.05) (data not shown).

Pilot studies revealed that combinations of trimethoprim or sertraline with levofloxacin gave the most promising results so these were selected for detailed study. A single treatment with combinations of levofloxacin + trimethoprim or levofloxacin + sertraline resulted in a small increase in survival of larvae infected with the parent strain (PAM1020) after 96 h compared to those treated with PBS or the constituent monotherapies but conferred no additional therapeutic benefit over the monotherapies with the strain over-expressing mexAB-oprM (PAM1032) (data not shown). The experiment was repeated using triple-dosing of each treatment 2, 4 and 6 h p.i (Figure 2). Triple doses of levofloxacin + trimethoprim or levofloxacin + sertraline resulted in significant increases in survival compared to triple doses of any of the monotherapies over the 96 h duration of the experiment. Notably, the enhanced therapeutic benefit conferred by treatment with the combinations was observed for infections with either the parent (PAM1020) or the strain over-expressing mexAB-oprM (PAM1032). This is in contrast to the results seen in vitro where neither of the combinations was observed to be synergistic when tested against the mexAB-oprM over-expressing strain.

Comparison of the effect of triple doses of levofloxacin + trimethoprim or levofloxacin + sertraline with the constituent monotherapies on the larval burden of the parent strain (PAM 1020) is shown in Figure 3. Larval burden following three doses of PBS increased from below the level of detection (≤ 2 log_{10} cfu/mL) 5 h post-infection to greater than 9 log_{10} cfu/mL after 24 h at 37°C. Treatment with three doses of trimethoprim (100mg/kg) or sertraline (100 mg/kg) also resulted in rapid proliferation of bacteria after 24 h. The rapid growth in bacterial numbers seen 24 h after triple-dose treatment with PBS, trimethoprim or sertraline...
correlated with death of the larval population (Figure 2). Treatment with three doses of levofloxacin (1 mg/kg) did retard bacterial growth after 24 h but this inhibition was abolished after 48 h as the bacterial population increased to approximately $10^{10}$ cfu/mL and the larval population died (Figure 2). Treatment with triple doses of levofloxacin + trimethoprim or levofloxacin + sertraline completely eradicated bacterial growth in infected larvae with detected numbers remaining below the level of detection ($\leq 2 \log_{10}$ cfu/mL) in most cases and this was reflected in the high levels of larval survival observed (Figure 2). In summary, the therapeutic benefit arising from treatment with efflux-pump inhibitor/antibiotic combination treatments correlates with reduced larval burden of infecting *P. aeruginosa*.

To determine if the enhanced therapeutic benefit arising from treatment with efflux-pump inhibitor/antibiotic combination treatments was also evident versus an infection by a different MDR strain an experiment was performed using *P. aeruginosa* NCTC13437 (Table 1). Importantly, treatment with triple doses of levofloxacin + trimethoprim or levofloxacin + sertraline again resulted in significant increases in survival of larvae infected with this strain compared to treatment with any of the constituent monotherapies over the 96 h duration of the experiment (Supplementary Figure 2).

**Discussion**

Strains of *P. aeruginosa* harbouring mutations that result in the over-expression of efflux-pumps are frequently isolated in the clinical setting. Invariably, these strains result in antibiotic treatment complications, or failure, due to their antibiotic-resistant phenotype. This study has demonstrated that *P. aeruginosa* strains that over-express efflux-pumps also result in the failure of antibiotic treatment in *G. mellonella* larvae. The similarity between the antibiotic-resistant phenotypes of strains in the invertebrate infection model and human patients shows that *G. mellonella* larvae represent a highly effective tool to develop and evaluate novel treatment options to target clinical MDR strains of *P. aeruginosa*.

Piddock *et al.* and Bohnert *et al.* presented evidence from *in vitro* studies that trimethoprim and sertraline both possessed EPI-like properties. Trimethoprim has been shown to be a substrate of the Mex pumps, in particular MexAB-OprM, and this explains the high levels of resistance measured in the over-expressing strains and the apparent susceptibility of the strain with all three Mex pumps deleted in this work (Supplementary Table 1). Data shown here (Table 3), reveals that combinations of antibiotics with
trimethoprim or sertraline that were synergistic versus the *P. aeruginosa* parent strain did not show synergy when applied to the strain with three efflux-pumps deleted supporting the previous conclusion that the two drugs do have EPI activity.

The present work has presented *in vivo* evidence that antibiotic and trimethoprim (or sertraline) combinations represent a potential treatment option for *P. aeruginosa* infections. Administration of combinations of trimethoprim or sertraline with levofloxacin conferred significant therapeutic benefit *in vivo* to infected *G. mellonella* larvae compared to those treated with the individual monotherapies. Importantly, the enhanced therapeutic effect of these combinations *in vivo* was demonstrated against a MDR strain of *P. aeruginosa* that over-expresses the MexAB-OprM efflux-pump. In terms of clinical relevance, the doses of levofloxacin (1-5 mg/kg) employed in this study are comparable to those used in humans – approximately 8 mg/kg. For human therapy, trimethoprim is usually administered in combination with sulfamethoxazole with trimethoprim at 20 mg/kg. This is lower than the dose of trimethoprim (100 mg/kg) in the successful combination reported here and further studies will be needed to determine if it is feasible to employ this higher dose of trimethoprim clinically. Furthermore, Bohnert *et al.* have already stated that sertraline is unlikely to be successful in combination treatments as the peak plasma levels attained are below the concentrations that are required to inhibit efflux.

It is notable that combinations of both trimethoprim and sertraline with levofloxacin were not synergistic versus the strain over-expressing the MexAB-OprM efflux-pump *in vitro* but resulted in significant therapeutic benefit when tested *in vivo*. Similarly, combinations of PAβN with levofloxacin were synergistic *in vitro* but conferred no therapeutic advantage over monotherapies when administered *in vivo* (data not shown). These discrepancies between results obtained *in vitro* with those observed *in vivo* concur with previous observations evaluating the efficacy of antibiotic only combination therapies carried out in the corresponding author’s lab. In fact, there are examples in the literature where synergy between two antibiotics observed *in vitro* does not translate into enhanced therapeutic benefit in patients. The implication of this is that by employing traditional *in vitro* methods to screen for effective combination treatments (or novel antimicrobials *per se*) researchers could actually be discarding potential treatments that are efficacious *in vivo*. Thus, screening *in vivo* using *G. mellonella* may be a better predictor of the efficacy of novel treatments in patients than *in vitro* assays. To evaluate this possibility, studies are needed urgently that compare the relative efficacy of established and novel treatments on infections in *G. mellonella* with murine models, and ultimately in human patients, to determine how relevant to the
outcomes of human infections results obtained with \( G. \) \( mellonella \) actually are. With \( P. \) \( aeruginosa \), a positive correlation between the degrees of pathogenicity of a range of mutant strains was demonstrated in \( G. \) \( mellonella \) and mice\(^2^3\) but there are few published studies comparing antibiotic efficacy in \( G. \) \( mellonella \) with murine, or human, infections. Clearly, any effects identified in \( G. \) \( mellonella \) may not be relevant to other mammalian infection models or humans. In addition to the obvious differences in the immune system of invertebrates and mammals, the efficacy of some antimicrobial drugs versus infected \( G. \) \( mellonella \) has been attributed not just to direct inhibition of the pathogen but also to activation, or priming, of the larval innate immune response.\(^2^4\) Additional experimentation will be required to determine if the efficacy of the combination treatments reported here is relevant in mammals.

In summary, antibiotic-resistant strains of \( P. \) \( aeruginosa \) that over-express drug efflux-pumps and result in treatment failure in human patients also result in the failure of antibiotic treatment in \( G. \) \( mellonella \) larvae. Furthermore, combination treatments consisting of the putative EPIs, trimethoprim and sertraline, with levofloxacin were significantly more efficacious than the individual monotherapies. The enhanced efficacy of these combinations versus infected \( G. \) \( mellonella \) was evident against a MDR strain of \( P. \) \( aeruginosa \) that over-expresses the MexAB-OprM efflux-pump, confirming that combinations of antibiotics with EPIs are a potential treatment option for real infections with MDR \( P. \) \( aeruginosa \). This study has also highlighted discrepancies in results obtained from in vitro and in vivo antimicrobial testing that could mean false positives are inadvertently selected and novel treatment options missed. Together, these findings further demonstrate the utility of using \( G. \) \( mellonella \) to develop and evaluate novel treatment options to target clinical MDR strains of \( P. \) \( aeruginosa \).

**Funding**

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**Transparency Declaration**

Nothing to declare.
References


9. Piddock LJV, Garvey MI, Mukhlesur Rahman M et al. Natural and synthetic compounds such as trimethoprim behave as inhibitors of efflux in Gram-negative bacteria. *J Antimicrob Chemother* 2010; **65**:1215-23.


**Legends for Figures**

**Figure 1.** Effect of antibiotic treatment on survival of *G. mellonella* larvae infected with *P. aeruginosa*

PAM1020, 1032, 1033, 1034 and 1626. Larvae were infected with $2.5 \times 10^3$ cfu/mL of each strain. A single dose of antibiotic was administered 2 h p.i.: LVX at 0.2, 1 or 5 mg/kg; PIP at 10, 25 or 100 mg/kg; and MER at 0.1, 0.25 or 0.5 mg/kg. Larvae were incubated at 37°C for 96 h and survival recorded every 24 h. The uninfected group represents larvae sham-infected with sterile PBS and treated with sterile PBS. The PBS group represents larvae infected with *P. aeruginosa* and treated with sterile PBS. For clarity, on each graph only data for PAM1020 is shown for the unmanipulated, uninfected and PBS control groups as the data was identical for each strain ($P>0.05$). * indicates a group with significantly enhanced or reduced survival in comparison to the other strains ($P<0.05$, log-rank test). $n=30$ (pooled from duplicate experiments).

**Figure 2.** Effect of treatment with combinations of LVX + TMP and LVX + SER on survival of *G. mellonella* larvae infected with *P. aeruginosa* PAM 1020 or PAM 1032. All larvae were inoculated with $2.5 \times 10^3$ cfu/mL *P. aeruginosa* and treated with each drug individually or in combination with three doses at 2, 4 and 6 h p.i. Treatments consisted of PBS, LVX, TMP (100 mg/kg) or SER (100 mg/kg) alone, or combinations of LVX with TMP (100 mg/kg) or SER (100 mg/kg). The LVX doses used were strain and dose-dependent and the dose is indicated on the graph (mg/kg). Larvae were incubated at 37°C for 96 h and survival recorded every 24 h. Data for groups of unmanipulated larvae is omitted as it was identical to the uninfected groups sham-infected with sterile PBS and treated with sterile PBS. * indicates a combination treatment group with significantly enhanced survival compared to any of the constituent monotherapies ($P<0.05$, log-rank test with Holm correction for multiple comparisons). $n=30$ (pooled from duplicate experiments).

**Figure 3.** Effect of treatment with combinations of LVX + TRIM and LVX + SER on larval burden of *P. aeruginosa* PAM1020. All larvae were inoculated with $2.5 \times 10^3$ cfu/mL *P. aeruginosa* and treated with three doses of each individual drug or combinations at 2, 4 and 6 h p.i. Treatments consisted of PBS, LVX (1 mg/kg), TMP (100 mg/kg) or SER (100 mg/kg) alone, or combinations of LVX (1 mg/kg) with TMP (100 mg/kg) or SER (100 mg/kg). Larvae were incubated at 37°C for 96 h and the burden of *P. aeruginosa* determined from 5 individual larvae every 24 h. For clarity, data for treatment with PBS, TMP and SER
alone is only shown for 24 h because the data obtained for subsequent data points closely followed that shown for LVX (1 mg/kg) treatment. * indicates a significant difference in larval burden between groups treated with the combination of LVX and TMP (or SER) compared to the constituent monotherapies; n=5 (* <0.05, Mann-Whitney U test). The black bar represents the median value of larval burden per group.
Table 1. *Pseudomonas aeruginosa* strains used in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Status of efflux pumps</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAM1020</td>
<td>PAO1 prototroph</td>
<td>Wild-type control</td>
<td>11</td>
</tr>
<tr>
<td>PAM1626</td>
<td>ΔmexAB-oprM::Cm; ΔmexCD-oprJ::Gm; ΔmexEF-oprN::ΩHg</td>
<td>mexAB-oprM; mexCD-oprJ; and mexEF-oprN deleted</td>
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<tr>
<td>PAM1033</td>
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<td>mexCD-oprJ over-expressed</td>
<td>11</td>
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<tr>
<td>PAM1034</td>
<td><em>nfxC</em> - type mutation</td>
<td>mexEF-oprN over-expressed</td>
<td>11</td>
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<tr>
<td>NCTC13437</td>
<td>Clinical isolate producing VEB-1 and VIM-10 β-lactamases</td>
<td>Resistant to fluoroquinolones and aminoglycosides by unknown mechanisms</td>
<td>12</td>
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Table 2. MICs of six antibiotics against *Pseudomonas aeruginosa* PAM1020, 1032, 1033, 1034 and 1626 determined in Mueller-Hinton broth after incubation at 37°C for 24 h. Each experiment was performed at least in triplicate.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype (^{a})</th>
<th>MIC (mg/L)</th>
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<tbody>
<tr>
<td></td>
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<td><em>nfxC</em></td>
<td>1</td>
</tr>
<tr>
<td>PAM1626</td>
<td>Δ<em>mex</em></td>
<td>0.5</td>
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</table>

\(^{a}\)WT, isogenic parent; *nalB*, overexpressing MexAB-OprM; *nfxB*, over-expressing MexCD-OprJ; *nfxC*, overexpressing MexEF-OprN; Δ*mex*, triple deletion Δ*mexAB oprM:*Cm; Δ*mexCD oprJ:*Gm; Δ*mexEF oprN:*ΩHg.
Table 3. MICs of three antibiotics in the absence and presence of three putative EPIs against *Pseudomonas aeruginosa* PAM1020, 1032 and 1626. Antibiotics were double diluted in Mueller-Hinton broth and EPI added to each well individually, plates were incubated for 24 h at 37°C. Each experiment was completed at least in duplicate.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Drug</th>
<th>Drug MIC (mg/L) with EPI</th>
<th>FIC index&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No EPI</td>
<td>TMP&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>PAM1020</td>
<td>LVX</td>
<td>0.5</td>
<td>&gt;0.004</td>
</tr>
<tr>
<td></td>
<td>PIP</td>
<td>4-8</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>MER</td>
<td>0.5-1</td>
<td>0.5</td>
</tr>
<tr>
<td>PAM1032</td>
<td>LVX</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>PIP</td>
<td>16-32</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>MER</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>PAM1626</td>
<td>LVX</td>
<td>0.031</td>
<td>0.031</td>
</tr>
<tr>
<td></td>
<td>PIP</td>
<td>0.25-0.5</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>MER</td>
<td>0.062-0.125</td>
<td>0.125</td>
</tr>
</tbody>
</table>

<sup>a</sup> The concentration of putative EPI added to each well reflected the previously characterised EPI MICs (Table 2) and were selected to be lower than the EPI MIC for each strain. PAM1020: TMP (50 mg/L), SER (50 mg/L), PAβN (100 mg/L); PAM1032 TMP (100 mg/L), SER (50 mg/L), PAβN (100 mg/L); PAM1626 TMP (0.5 mg/L), SER (5 mg/L), PAβN (10 mg/L).

<sup>b</sup> Fractional inhibitory concentration index (FIC index) where synergistic (≤ 0.5), non-synergistic (> 0.5 – ≤4.0) and antagonistic (>4). Where actual MICs were not detected for some EPIs, the highest value tested was used in the FICI calculation to provide a conservative estimate of the FICI value. Bold text indicates synergy.
Figure 1

LVX
Unmanipulated
Uninfected

0.2 mg/kg

0.25 mg/kg

0.5 mg/kg

Time (h)

PAM1626

PBS

PIP
Unmanipulated
Uninfected

1 mg/kg

25 mg/kg

Time (h)

PAM1626

PAM1034

PAM1033

PAM1020

MER
Unmanipulated
Uninfected

0.1 mg/kg

0.25 mg/kg

0.5 mg/kg

Time (h)

PAM1626

PAM1032

PAM1033

PAM1020

PAM1034

PBS
Figure 2

The figure depicts survival rates over time for different conditions. The x-axis represents time in hours (0 to 96), and the y-axis represents the percentage of survivors. Two sets of conditions are shown:

1. PAM1020:
   - PBS/PBS
   - Infected/LVX 1 + TMP 100
   - Infected/LVX 1 + SER 100
   - Infected/TMP 100
   - Infected/SER 100
   - Infected/PBS

2. PAM1032:
   - PBS/PBS
   - Infected/LVX 5 + TMP 100
   - Infected/LVX 5 + SER 100
   - Infected/TMP 100
   - Infected/SER 100
   - Infected/PBS
Figure 3

Larval burden (Log₁₀ cfu/mL) vs. Time (h)

- Δ LVX 1 mg/kg
- ◇ LVX 1 mg/kg + TMP 100 mg/kg
- □ LVX 1 mg/kg + SER 100 mg/kg
- ■ PBS
- ● TMP 100 mg/kg
- ○ SER 100 mg/kg