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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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23 **Synopsis**

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

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

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

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

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49 Introduction

50 *Pseudomonas aeruginosa* is a ubiquitous, opportunistic pathogen prominent in hospitalised,
51 immunocompromised patients where it targets the urinary and respiratory tracts, kidneys and bloodstream
52 principally via invasive fomites such as catheters and mechanical ventilators.¹ Treatment of *P. aeruginosa*
53 infections can be difficult due to its high intrinsic resistance, which in turn has been amplified by the rapid
54 spread of resistance determinants and arrival of the MDR phenotype where isolates are resistant to three
55 or more different classes of antibiotics.

56 The over-expression of membrane-associated efflux-pumps that contribute to reduced efficacy of a number
57 of important classes of antibiotics used against *P. aeruginosa* is an important contributor to resistance.²

58 The most significant family of efflux-pumps that mediate antibiotic resistance in *P. aeruginosa* are those
59 belonging to the resistance-nodulation-division (RND). Four of the twelve member RND family are strongly
60 associated with antibiotic efflux: MexAB-OprM, MexXY-OprM, MexCD-OprJ and MexEF-OprN.³ These four
61 pumps transport an overlapping range of antibiotic substrates, in particular the fluoroquinolones. However,
62 MexAB-OprM is the least discriminatory, with a broad substrate range allowing extrusion of almost all
63 classes of antibiotic, and is the most important efflux-pump mediating antibiotic resistance because it is
64 constitutively expressed and responsible for much of the intrinsic resistance of the organism.³ Knockout of
65 mexAB-oprM results in hyper-susceptibility to antibiotics.⁴ In contrast, expression of MexCD-OprJ and
66 MexEF-OprN requires exposure to a broad range of compounds or environmental stimuli.² MexCD-OprJ's
67 substrate range mirrors that of MexAB-OprM while MexEF-OprN appears the least versatile.³ Knockout of
68 either of these two pumps does not affect antibiotic susceptibility.⁵ Mutations in efflux-pump regulatory
69 genes can result in over-expression of any of these pumps and confer a multi-drug resistant phenotype.
70 Importantly, these mutations are commonly identified in clinical isolates, for example, mutations in *nalB*,⁶
71 *nfxB*⁷ and *nfxC*⁸ result in over-expression of MexAB-OprM, MexCD-OprJ and MexEF-OprN respectively.

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

75 In two independent studies, evidence was presented that the antibiotic trimethoprim⁹ and the selective
76 serotonin reuptake inhibitor sertraline¹⁰ were synergistic in combination with a range of antibiotics versus
77 Gram-negative bacteria *in vitro*. Both drugs demonstrated EPI-like activity by inhibiting the efflux of dyes

78 from these bacteria. Using a collection of characterised *P. aeruginosa* strains that over-express three RND
79 efflux pumps (MexAB-OprM, MexCD-OprJ, MexEF-OprN¹¹), the aim of this study was to assess the efficacy
80 of combinations of antibiotics with sertraline and trimethoprim using a *Galleria mellonella* infection model *in*
81 *vivo* to determine if these combinations could represent a future treatment option for infections with *P.*
82 *aeruginosa* strains that display an efflux-pump mediated MDR phenotype.

84 **Materials and Methods**

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

91 *Antibiotics and G. mellonella larvae.* All antibiotics: ceftazidime (CFD), piperacillin (PIP), meropenem
92 (MER), amikacin (AMK), levofloxacin (LVX) and colistin (CST) and Pseudomonas Isolation Agar (PIA) were
93 purchased from Sigma-Aldrich Ltd (Dorset, UK). Stock solutions and sub-stocks were made using sterile
94 deionised water. Putative efflux-pump inhibitors sertraline HCl (SER), trimethoprim (TMP) and PAβN were
95 purchased from Sigma-Aldrich Ltd. Stock solutions of sertraline and trimethoprim were made in 50% and
96 75% (v/v) DMSO respectively. PAβN was made in sterile deionised water. Sub-stocks of all drugs for use in
97 experiments were made in sterile deionised water. *G. mellonella* larvae were purchased from UK
98 Waxworms Ltd (Sheffield, UK).

99 *Drug susceptibility testing.* This was performed exactly as previously described.¹³ Briefly, the MIC of each
100 antibiotic and the putative EPIs versus *P. aeruginosa* strains was determined in 96-well microplates
101 (Greiner Bio-one Ltd, Stonehouse, UK) via doubling dilution of each drug in MHB and subsequent
102 inoculation with 1.0x10⁶ cfu/mL of *P. aeruginosa*. The MIC value of combinations of antibiotics with EPIs
103 was carried out exactly as above using 96-well microplate assays prepared via doubling dilution of each
104 antibiotic in MHB followed by addition of a single concentration of each EPI. All three putative EPIs
105 (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,¹⁴ 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.¹³ Unless otherwise stated, groups of larvae were infected with an inoculum of 2.5×10^3 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 $P \leq 0.05$ was considered significant and Holm's correction was always applied to account for multiple comparisons.¹⁵

For haemolymph burden, groups of 30 larvae were infected with 2.5×10^3 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.¹⁶

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

134 pump expression and the isogenic parent strain (Supplementary Figure 1). With all strains tested, the heat-
135 killed *P. aeruginosa* inoculum (inoculation dose of 2.5×10^8 cfu/mL) had no significant detrimental effect on
136 larval survival ($P > 0.05$) indicating that infection with live bacteria is required to cause larval death.
137 Inoculation with 25 cfu/larva of each strain resulted in 100% lethality after 24 h compared to controls sham-
138 infected with PBS. No difference in virulence between the strains was discernible. Reducing the inoculum
139 size 10-fold to 2.5 cfu/larva allowed better discrimination of any differences in virulence between the
140 strains. Despite some apparent differences in larval survival 48 h post-infection (p.i), after 96 h there was
141 no significant difference ($P \geq 0.05$) in survival of larvae infected with any of the strains tested. In conclusion,
142 alteration of efflux-pump expression had no significant effect on the virulence of *P. aeruginosa* in *G.*
143 *mellonella* larvae.

144
145 *Antibiotic susceptibility of P. aeruginosa strains with altered expression levels of efflux-pumps is affected in*
146 *vitro and this correlates with the efficacy of these antibiotics versus G. mellonella larvae infected with the*
147 *same strains. In vitro* susceptibility of the parent strain and four efflux-pump mutants to a range of anti-
148 Pseudomonal antibiotics is shown in Table 2. Over-expression of *mexAB-oprM* (PAM1032) conferred
149 resistance to ceftazidime, piperacillin, meropenem and levofloxacin compared to the isogenic parent
150 (PAM1020). Over-expression of *mexCD-oprJ* (PAM1033) and *mexEF-oprN* (PAM1034) conferred
151 significant resistance to levofloxacin only. The mutant with all three efflux pumps deleted (PAM1626)
152 displayed susceptibility to ceftazidime, piperacillin, meropenem and levofloxacin compared to the parent
153 (PAM1020). Together, these results are consistent with many previous studies on the substrate specificity
154 of these efflux-pumps.²

155 To study the effect of altered efflux pump expression on *in vivo* antibiotic efficacy infected larvae were
156 treated with levofloxacin, piperacillin and meropenem as these three drugs had the largest differences in
157 susceptibility between the mutant and parent strains *in vitro* (see Table 2). Larvae were infected with each
158 strain of *P. aeruginosa* and the effect of a single, increasing dose of each antibiotic on survival measured
159 (Figure 1). Treatment with 0.2 mg/kg of levofloxacin conferred no therapeutic benefit on larvae infected with
160 any of the *P. aeruginosa* strains tested. Notably, a single dose of 1 mg/kg levofloxacin only induced
161 therapeutic benefit on larvae infected with the mutant with all three efflux pumps deleted (PAM1626).
162 Increasing the dose to 5 mg/kg levofloxacin resulted in almost complete survival of larvae infected with the

163 triple deletion mutant but also the parent strain (PAM1020). Notably, this highest dose of levofloxacin
164 offered no therapeutic benefit to larvae infected with the three mutant strains that over-expressed the three
165 efflux pumps (PAM1032, PAM1033 and PAM1034). A similar trend of antibiotic efficacy, dependent on the
166 level of efflux-pump expression, was also observed when larvae infected with each strain were treated with
167 increasing doses of piperacillin. Similar to treatment with levofloxacin and piperacillin, treatment with
168 meropenem was most effective on larvae infected with the mutant with all three efflux pumps deleted
169 (PAM1626). Treatment with 0.25 or 0.5 mg/kg meropenem proved to be efficacious to larvae infected with
170 the parent strain (PAM1020) and the strains over-expressing *mexCD-oprJ* (PAM1033) or *mexEF-oprN*
171 (PAM1034). Notably, over-expression of *mexAB-oprM* (PAM1032) abolished this efficacy.

172 In conclusion, the expression level of efflux-pumps clearly influences antibiotic efficacy *in vivo*.

173 Furthermore, the *in vivo* efficacy of levofloxacin, piperacillin and meropenem versus larvae infected with *P.*
174 *aeruginosa* strains displaying altered expression of efflux-pumps reflected exactly the degree of
175 susceptibility to the same drugs that was measured *in vitro*.

176
177 *Treatment of G. mellonella larvae infected with MDR strains of P. aeruginosa with a combination of putative*
178 *efflux-pump inhibitors and levofloxacin results in enhanced therapeutic benefit compared to the constituent*
179 *monotherapies*. The antibiotic trimethoprim⁹ and the selective serotonin reuptake inhibitor sertraline¹⁰
180 represent two clinically-approved drugs that have also been shown to be putative inhibitors of Gram-
181 negative efflux-pumps. The MICs of these two drugs and the established EPI PA β N¹⁷ are shown in
182 Supplementary Table 1. Only the mutant with all three efflux-pumps deleted (PAM1626) displayed any
183 substantial susceptibility, and the three strains over-expressing individual efflux pumps had a higher MIC
184 for trimethoprim than the parent, implying that all three putative EPIs may be substrates of these Mex
185 efflux-pumps. MIC and FICI values of the three EPIs in combination with levofloxacin, piperacillin and
186 meropenem are shown in Table 3. Combination MICs were determined for the parent strain (PAM1020),
187 the triple efflux-pump deletion (PAM1626) and the mutant over-expressing *mexAB-oprM* (PAM1032).
188 PAM1032 was included over the mutants over-expressing *mexCD-oprJ* and *mexEF-oprN* because this
189 strain was the most resistant to levofloxacin, piperacillin and meropenem treatment *in vivo* and MexAB-
190 oprM is responsible for intrinsic resistance of *P. aeruginosa* to many antibiotics. Combination of
191 trimethoprim, sertraline and PA β N with levofloxacin each resulted in a reduction in the MIC of levofloxacin

192 versus the parent strain (PAM1020). The calculated FICI values for each of these combinations indicated
193 synergistic inhibition (≤ 0.5). Synergy was also observed against the parent strain for combinations of
194 piperacillin with trimethoprim and PA β N, and meropenem with sertraline. None of the putative EPI/antibiotic
195 combinations showed significant synergy versus the strain with all three efflux-pumps deleted (PAM1626).
196 In contrast to the parent strain (PAM1020), trimethoprim and sertraline did not act in synergy with the
197 antibiotics versus the mutant over-expressing *mexAB-oprM* (PAM1032). Only the established efflux-pump
198 inhibitor PA β N was synergistic in combination with all three antibiotics against this mutant.

199 Antibiotic and EPI combinations were then investigated in the *G. mellonella* infection model for *in vivo*
200 efficacy. Irrespective of the *P. aeruginosa* strain investigated, treatment of infected larvae with PA β N (at
201 100mg/kg) in combination with levofloxacin or piperacillin (both at strain specific doses) did not confer any
202 therapeutic benefit over antibiotic treatment alone ($P \geq 0.05$) (data not shown).

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

215 Comparison of the effect of triple doses of levofloxacin + trimethoprim or levofloxacin + sertraline with the
216 constituent monotherapies on the larval burden of the parent strain (PAM 1020) is shown in Figure 3. Larval
217 burden following three doses of PBS increased from below the level of detection ($\leq 2 \log_{10}$ cfu/mL) 5 h post-
218 infection to greater than $9 \log_{10}$ cfu/mL after 24 h at 37°C. Treatment with three doses of trimethoprim
219 (100mg/kg) or sertraline (100 mg/kg) also resulted in rapid proliferation of bacteria after 24 h. The rapid
220 growth in bacterial numbers seen 24 h after triple-dose treatment with PBS, trimethoprim or sertraline

221 correlated with death of the larval population (Figure 2). Treatment with three doses of levofloxacin (1
222 mg/kg) did retard bacterial growth after 24 h but this inhibition was abolished after 48 h as the bacterial
223 population increased to approximately $10 \log_{10}\text{cfu/mL}$ and the larval population died (Figure 2). Treatment
224 with triple doses of levofloxacin + trimethoprim or levofloxacin + sertraline completely eradicated bacterial
225 growth in infected larvae with detected numbers remaining below the level of detection ($\leq 2 \log_{10}\text{cfu/mL}$) in
226 most cases and this was reflected in the high levels of larval survival observed (Figure 2). In summary, the
227 therapeutic benefit arising from treatment with efflux-pump inhibitor/antibiotic combination treatments
228 correlates with reduced larval burden of infecting *P. aeruginosa*.

229 To determine if the enhanced therapeutic benefit arising from treatment with efflux-pump inhibitor/antibiotic
230 combination treatments was also evident versus an infection by a different MDR strain an experiment was
231 performed using *P. aeruginosa* NCTC13437¹² (Table 1). Importantly, treatment with triple doses of
232 levofloxacin + trimethoprim or levofloxacin + sertraline again resulted in significant increases in survival of
233 larvae infected with this strain compared to treatment with any of the constituent monotherapies over the 96
234 h duration of the experiment (Supplementary Figure 2).

236 Discussion

237 Strains of *P. aeruginosa* harbouring mutations that result in the over-expression of efflux-pumps are
238 frequently isolated in the clinical setting. Invariably, these strains result in antibiotic treatment complications,
239 or failure, due to their antibiotic-resistant phenotype.^{18,19} This study has demonstrated that *P. aeruginosa*
240 strains that over-express efflux-pumps also result in the failure of antibiotic treatment in *G. mellonella*
241 larvae. The similarity between the antibiotic-resistant phenotypes of strains in the invertebrate infection
242 model and human patients shows that *G. mellonella* larvae represent a highly effective tool to develop and
243 evaluate novel treatment options to target clinical MDR strains of *P. aeruginosa*.

244 Piddock *et al.*⁹ and Bohnert *et al.*¹⁰ presented evidence from *in vitro* studies that trimethoprim and sertraline
245 both possessed EPI-like properties. Trimethoprim has been shown to be a substrate of the Mex pumps, in
246 particular MexAB-OprM²⁰, and this explains the high levels of resistance measured in the over-expressing
247 strains and the apparent susceptibility of the strain with all three Mex pumps deleted in this work
248 (Supplementary Table 1). Data shown here (Table 3), reveals that combinations of antibiotics with

249 trimethoprim or sertraline that were synergistic versus the *P. aeruginosa* parent strain did not show synergy
250 when applied to the strain with three efflux-pumps deleted supporting the previous conclusion that the two
251 drugs do have EPI activity.

252 The present work has presented *in vivo* evidence that antibiotic and trimethoprim (or sertraline)
253 combinations represent a potential treatment option for *P. aeruginosa* infections. Administration of
254 combinations of trimethoprim or sertraline with levofloxacin conferred significant therapeutic benefit *in vivo*
255 to infected *G. mellonella* larvae compared to those treated with the individual monotherapies. Importantly,
256 the enhanced therapeutic effect of these combinations *in vivo* was demonstrated against a MDR strain of *P.*
257 *aeruginosa* that over-expresses the MexAB-OprM efflux-pump. In terms of clinical relevance, the doses of
258 levofloxacin (1-5 mg/kg) employed in this study are comparable to those used in humans – approximately 8
259 mg/kg.¹³ For human therapy, trimethoprim is usually administered in combination with sulfamethoxazole
260 with trimethoprim at 20 mg/kg.²¹ This is lower than the dose of trimethoprim (100 mg/kg) in the successful
261 combination reported here and further studies will be needed to determine if it is feasible to employ this
262 higher dose of trimethoprim clinically. Furthermore, Bohnert *et al.*¹⁰ have already stated that sertraline is
263 unlikely to be successful in combination treatments as the peak plasma levels attained are below the
264 concentrations that are required to inhibit efflux.

265 It is notable that combinations of both trimethoprim and sertraline with levofloxacin were not synergistic
266 versus the strain over-expressing the MexAB-OprM efflux-pump *in vitro* but resulted in significant
267 therapeutic benefit when tested *in vivo*. Similarly, combinations of PA β N with levofloxacin were synergistic
268 *in vitro* but conferred no therapeutic advantage over monotherapies when administered *in vivo* (data not
269 shown). These discrepancies between results obtained *in vitro* with those observed *in vivo* concur with
270 previous observations evaluating the efficacy of antibiotic only combination therapies carried out in the
271 corresponding author's lab.¹⁶ In fact, there are examples in the literature where synergy between two
272 antibiotics observed *in vitro* does not translate into enhanced therapeutic benefit in patients.²² The
273 implication of this is that by employing traditional *in vitro* methods to screen for effective combination
274 treatments (or novel antimicrobials *per se*) researchers could actually be discarding potential treatments
275 that are efficacious *in vivo*. Thus, screening *in vivo* using *G. mellonella* may be a better predictor of the
276 efficacy of novel treatments in patients than *in vitro* assays. To evaluate this possibility, studies are needed
277 urgently that compare the relative efficacy of established and novel treatments on infections in *G.*
278 *mellonella* with murine models, and ultimately in human patients, to determine how relevant to the

279 outcomes of human infections results obtained with *G. mellonella* actually are. With *P. aeruginosa*, a
280 positive correlation between the degrees of pathogenicity of a range of mutant strains was demonstrated in
281 *G. mellonella* and mice²³ but there are few published studies comparing antibiotic efficacy in *G. mellonella*
282 with murine, or human, infections. Clearly, any effects identified in *G. mellonella* may not be relevant to
283 other mammalian infection models or humans. In addition to the obvious differences in the immune system
284 of invertebrates and mammals, the efficacy of some antimicrobial drugs versus infected *G. mellonella* has
285 been attributed not just to direct inhibition of the pathogen but also to activation, or priming, of the larval
286 innate immune response.²⁴ Additional experimentation will be required to determine if the efficacy of the
287 combination treatments reported here is relevant in mammals.

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

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302

303 **Transparency Declaration**

304 Nothing to declare.
305

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378 **Legends for Figures**

379 **Figure 1.** Effect of antibiotic treatment on survival of *G. mellonella* larvae infected with *P. aeruginosa*
380 PAM1020, 1032, 1033, 1034 and 1626. Larvae were infected with 2.5×10^3 cfu/mL of each strain. A single
381 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
382 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
383 uninfected group represents larvae sham-infected with sterile PBS and treated with sterile PBS. The PBS
384 group represents larvae infected with *P. aeruginosa* and treated with sterile PBS. For clarity, on each graph
385 only data for PAM1020 is shown for the unmanipulated, uninfected and PBS control groups as the data
386 was identical for each strain ($P>0.05$). * indicates a group with significantly enhanced or reduced survival in
387 comparison to the other strains ($P<0.05$, log-rank test). $n=30$ (pooled from duplicate experiments).

388

389 **Figure 2.** Effect of treatment with combinations of LVX + TMP and LVX + SER on survival of *G. mellonella*
390 larvae infected with *P. aeruginosa* PAM 1020 or PAM 1032. All larvae were inoculated with 2.5×10^3 cfu/mL
391 *P. aeruginosa* and treated with each drug individually or in combination with three doses at 2, 4 and 6 h p.i.
392 Treatments consisted of PBS, LVX, TMP (100 mg/kg) or SER (100 mg/kg) alone, or combinations of LVX
393 with TMP (100 mg/kg) or SER (100 mg/kg). The LVX doses used were strain and dose-dependent and the
394 dose is indicated on the graph (mg/kg). Larvae were incubated at 37°C for 96 h and survival recorded every
395 24 h. Data for groups of unmanipulated larvae is omitted as it was identical to the uninfected groups sham-
396 infected with sterile PBS and treated with sterile PBS. * indicates a combination treatment group with
397 significantly enhanced survival compared to any of the constituent monotherapies ($P<0.05$, log-rank test
398 with Holm correction for multiple comparisons). $n=30$ (pooled from duplicate experiments).

399

400 **Figure 3.** Effect of treatment with combinations of LVX + TRIM and LVX + SER on larval burden of *P.*
401 *aeruginosa* PAM1020. All larvae were inoculated with 2.5×10^3 cfu/mL *P. aeruginosa* and treated with three
402 doses of each individual drug or combinations at 2, 4 and 6 h p.i. Treatments consisted of PBS, LVX (1
403 mg/kg), TMP (100 mg/kg) or SER (100 mg/kg) alone, or combinations of LVX (1 mg/kg) with TMP (100
404 mg/kg) or SER (100 mg/kg). Larvae were incubated at 37°C for 96 h and the burden of *P. aeruginosa*
405 determined from 5 individual larvae every 24 h. For clarity, data for treatment with PBS, TMP and SER

406 alone is only shown for 24 h because the data obtained for subsequent data points closely followed that
407 shown for LVX (1 mg/kg) treatment. # indicates a significant difference in larval burden between groups
408 treated with the combination of LVX and TMP (or SER) compared to the constituent monotherapies; n=5
409 ($P<0.05$, Mann-Whitney U test). The black bar represents the median value of larval burden per group.

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433 **Table 1.** *Pseudomonas aeruginosa* strains used in this study.

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Strain	Genotype	Status of efflux pumps	Reference
PAM1020	PAO1 prototroph	Wild-type control	11
PAM1626	$\Delta mexAB-oprM::Cm$; $\Delta mexCD-oprJ::Gm$; $\Delta mexEF-oprN::\Omega Hg$	<i>mexAB-oprM</i> ; <i>mexCD-oprJ</i> ; and <i>mexEF-oprN</i> deleted	11
PAM1032	<i>nalB</i> - type mutation	<i>mexAB-oprM</i> over-expressed	11
PAM1033	<i>nfxB</i> - type mutation	<i>mexCD-oprJ</i> over-expressed	11
PAM1034	<i>nfxC</i> - type mutation	<i>mexEF-oprN</i> over-expressed	11
NCTC13437	Clinical isolate producing VEB-1 and VIM-10 β -lactamases	Resistant to fluoroquinolones and aminoglycosides by unknown mechanisms	12

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459 **Table 2.** MICs of six antibiotics against *Pseudomonas aeruginosa* PAM1020, 1032, 1033, 1034 and 1626
460 determined in Mueller-Hinton broth after incubation at 37°C for 24 h. Each experiment was performed at
461 least in triplicate.

Strain	Genotype ^a	MIC (mg/L)					
		CFD	PIP	MER	LVX	AMK	CST
PAM1020	Parent	1	4-8	0.5-1	0.5	2	1-4
PAM1032	<i>nalB</i>	2-4	16-32	4	2	0.5-1	0.25-1
PAM1033	<i>nfxB</i>	0.5-1	2-4	0.25-1	4	0.25-1	0.5-1
PAM1034	<i>nfxC</i>	1	2	0.25-0.5	4	0.25-1	0.125-0.5
PAM1626	Δmex	0.5	0.25-0.5	0.0625-0.125	0.03125	1	1

464 ^aWT, isogenic parent; *nalB*, overexpressing MexAB-OprM; *nfxB*, over-expressing MexCD-OprJ; *nfxC*,
465 overexpressing MexEF-OprN; Δmex , triple deletion $\Delta mexAB-oprM::Cm$; $\Delta mexCD-oprJ::Gm$; $\Delta mexEF-$
466 *oprN::ΩHg*.
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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.

Strain	Drug	Drug MIC (mg/L) with				FIC index ^b		
		No EPI	TMP ^a	SER ^a	PAβN ^a	Drug+TMP	Drug+SER	Drug+PAβN
PAM1020	LVX	0.5	>0.004	0.125	0.156	0.008	0.252	0.031
	PIP	4-8	1	1-2	1-2	0.266	0.531	0.266
	MER	0.5-1	0.5	>0.004	0.25	0.508	0.008	0.502
PAM1032	LVX	2	2	2	0.031	1	1	0.016
	PIP	16-32	32	16	4	2	0.75	0.156
	MER	4	4	2	0.25	1	0.531	0.065
PAM1626	LVX	0.031	0.031	0.016	0.016	1	0.501	0.500
	PIP	0.25-0.5	0.5	0.25	0.5	2.25	0.516	1
	MER	0.062-0.125	0.125	0.062	0.125	1.1	1	1

^a 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).

^b Fractional inhibitory concentration index (FIC index) where synergistic (≤ 0.5), non-synergistic ($> 0.5 - \leq 4.0$) 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

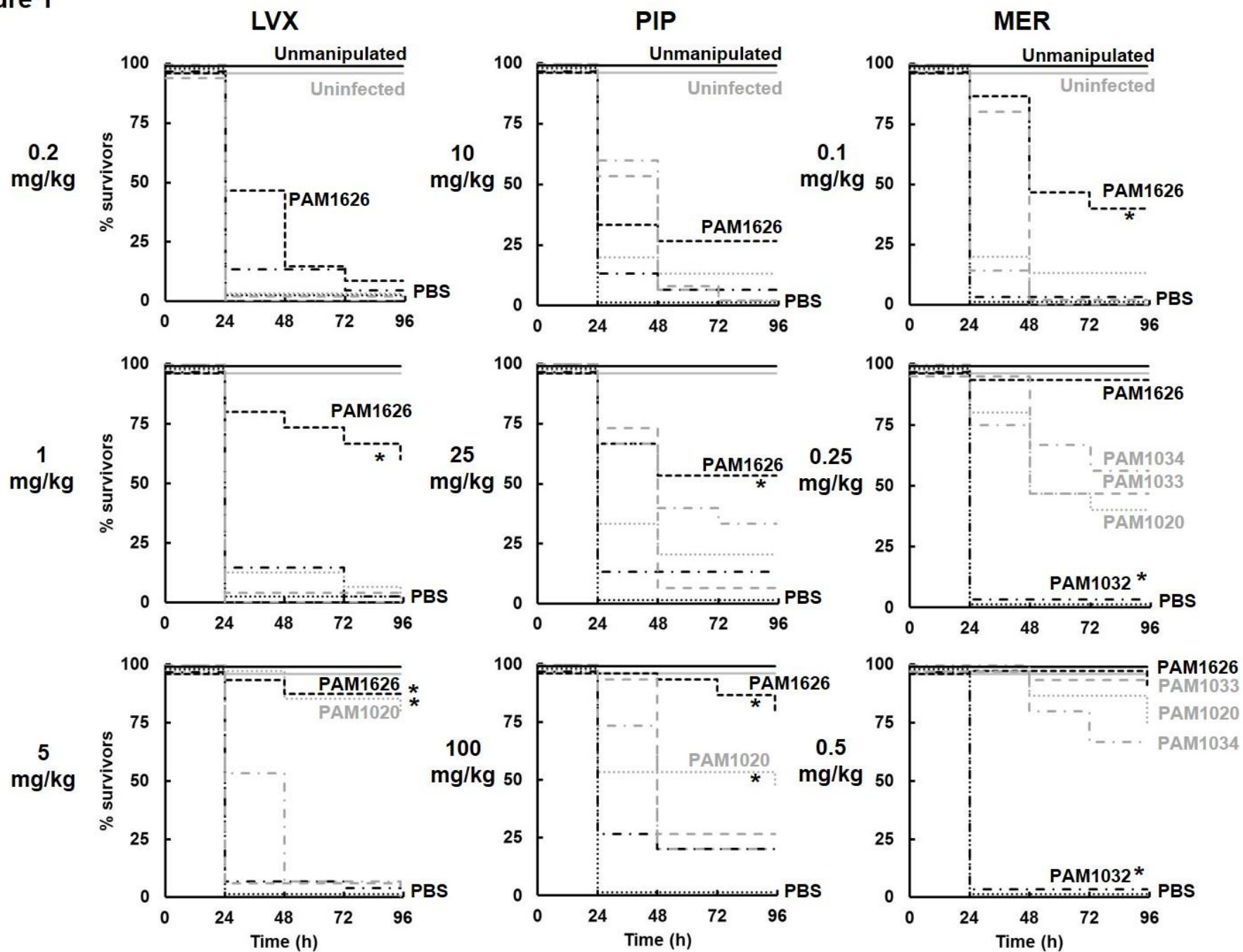
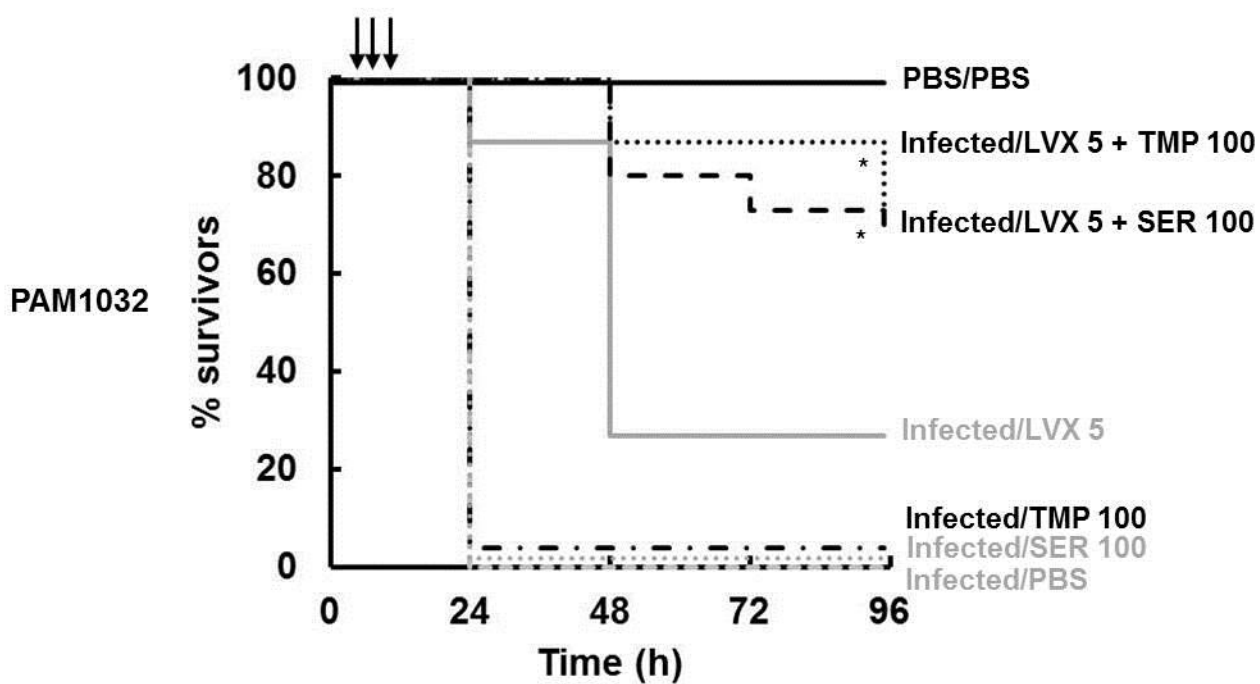
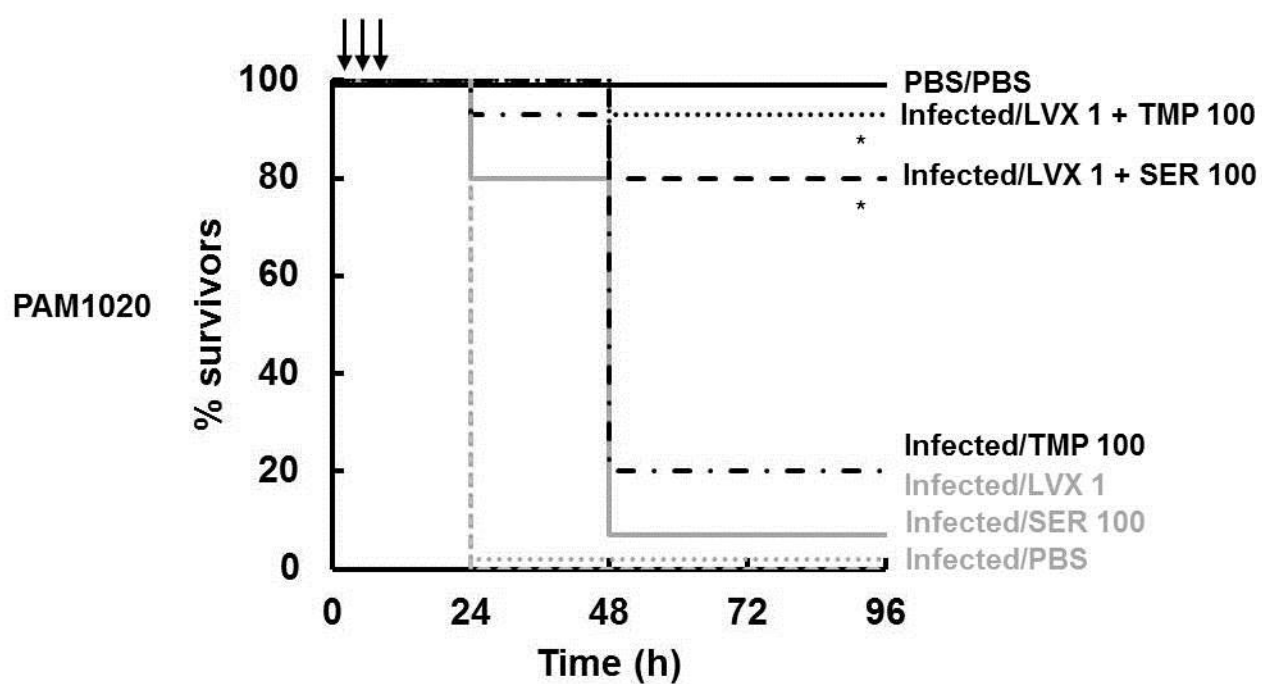


Figure 2



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Figure 3

