# **Permeability enhancers sensitize β-lactamase-expressing *Enterobacteriaceae* and *Pseudomonas aeruginosa* to β-lactam inhibitors thereby restoring their β-lactam susceptibility**

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24 **ABSTRACT**

25 β-lactamases are the major resistance determinant for β-lactam antibiotics in
26 Gram-negative bacteria. Although there are β-lactamase inhibitors (BLIs)
27 available, β-lactam-BLI combinations are increasingly being neutralized by
28 diverse mechanisms of bacterial resistance. We hypothesized that permeability-
29 increasing antimicrobial peptides (AMPs) could lower the amount of BLIs
30 necessary to sensitize bacteria to antibiotics that are β-lactamase substrates. To
31 test this hypothesis, we performed checkerboard assays and measured the ability
32 of several AMPs, to synergize with piperacillin, ticarcillin, amoxicillin, ampicillin
33 and ceftazidime in the presence of either, tazobactam, clavulanic acid,
34 sulbactam, aztreonam, phenylboronic acid (PBA) or oxacillin. Assays were
35 performed using planktonic and biofilm-forming cells of *Pseudomonas*
36 *aeruginosa*, *Escherichia coli* and *Klebsiella pneumoniae* strains overexpressing
37 β-lactamases. Synergy between polymyxin B nonapeptide (PMBN) and
38 tazobactam boosted piperacillin inhibitory activity by a factor of 128 in *E. coli* (from
39 256 to 2 mg/L, FICI=0.02) and by a factor of 64 in *K. pneumoniae* (from 1024
40 mg/L to 16 mg/L, FICI=0.05). Synergy between PMBN and PBA enhanced
41 ceftazidime activity 133 times in *P. aeruginosa* (from 16 mg/L to 0.12 mg/L,
42 FICI=0.03). As a consequence, MICs of all the antibiotics tested were brought
43 down to therapeutic range. In addition, the combinations also reduced several
44 orders of magnitude the amount of inhibitor needed for antibiotic sensitization.
45 Ceftazidime/PBA/PMBN at 50 times the planktonic MIC caused a 10 million-fold
46 reduction in the viability of a mature biofilm. We proved that AMPs can synergize
47 with BLIs and that this phenomenon can be exploited to sensitize bacteria to
48 antibiotics.

49 Keywords: Synergy; β-lactamase inhibitor; *Pseudomonas aeruginosa*;
50 *Escherichia coli*; *Klebsiella pneumoniae*; biofilm.

51 **1. INTRODUCTION**

52 Emergence of antibiotic resistance among bacterial pathogens is one of the major
53 health threats worldwide. Bacteria display several mechanisms of resistance that
54 neutralize antibiotic activity. One of them involves the production of β-lactamases,
55 enzymes that hydrolyze and inactivate chemical compounds containing a β-
56 lactam ring, such as β-lactam antibiotics. β-lactamases are the major resistance
57 determinant for β-lactam antibiotics in Gram-negative bacteria. They were first
58 described in 1940 and currently, the group encompasses over 2800 enzymes
59 [1,2].

60 At the clinical level, the most important β-lactamases expressed by *Escherichia*
61 *coli* and *Klebsiella pneumoniae* are the, so called, extended spectrum β-
62 lactamases (ESBLs) [3]. ESBLs are plasmid-encoded class A β-lactamases that
63 can hydrolyze penicillins, cephalosporins of $1^{st}$, $2^{nd}$, $3^{rd}$ and $4^{th}$ generation and
64 monobactams. In 2017, 87.4% and 87.8% of third-generation cephalosporin-
65 resistant *E. coli* and *K. pneumoniae* isolated in Europe were ESBL-positive,
66 respectively [4].

67 In the case of *Pseudomonas aeruginosa*, the most prevalent β-lactamase is
68 AmpC, an inducible class C chromosomal enzyme that is located in the bacterial
69 periplasm. Although, this enzyme is active against penicillins and monobactams,
70 it particularly excels in its ability to hydrolyze cephalosporins. AmpC has a low
71 level of constitutive expression and this allows *P. aeruginosa* to inactivate
72 aminopenicillins (e.g. amoxicillin and ampicillin) and cephalosporins of narrow

73 spectrum (i.e. of 1st and 2nd generation) under non-inducing conditions. However,
74 this pathogen can increase the production of AmpC between 100 and 1000 times
75 in the presence of inducers [5] and can reach a constitutive over-expression as a
76 result of mutations in regulator genes like *ampD* and *ampR* [6].

77 In the fight against β-lactamases, the development of β-lactamase inhibitors
78 (BLIs) was a major therapeutic breakthrough [7,8][9,10]. Tazobactam, sulbactam
79 and clavulanic acid are compounds with ESBL inhibitory activity that are currently
80 coadministered with piperacillin, ampicillin and amoxicillin. In turn, oxacillin,
81 aztreonam and boronic acid based compounds are efficient AmpC inhibitors [11–
82 13][14][15][16,17][18]. The cyclic borate vaborbactam has recently been
83 approved for clinical use in combination with meropenem [12,19–21].

84 Despite their long and successful therapeutic record, β-lactam-BLI combinations
85 are increasingly being neutralized by diverse mechanisms of bacterial resistance.
86 On the one hand, mutations can render β-lactamases resistant to BLIs [22].
87 Furthermore, bacteria can overexpress β-lactamases with low level of sensitivity
88 to BLIs (such as TEM-1) [23]. On the other hand, antibiotic resistance to BLIs
89 frequently arises from decreased membrane permeability or efflux mechanisms
90 which prevent the proper interaction between BLIs and β-lactamases [24].

91 To safeguard the clinical use of β-lactams-BLIs combinations, strategies that
92 restore the antimicrobial activity of inhibitors are urgently needed. In this context,
93 we hypothesized that permeabilization of outer membrane by antimicrobial
94 peptides (AMPs) could enhance BLI activity by allowing accumulation of the
95 inhibitor in the proximity of β-lactamases (i.e. in the bacterial periplasm). Our
96 group has successfully applied this strategy to potentiate the activity of efflux

97 pump inhibitors against *P. aeruginosa,* thereby sensitizing this pathogen to
98 macrolides, tetracyclines, quinolones, and β-lactam antibiotics [25]. In this study,
99 we will test whether this strategy is applicable to BLIs.

100 **2. MATERIAL AND METHODS**

101 **2.1. Culture conditions and susceptibility testing**

102 The *P. aeruginosa* strains used in this study (Table 1) were the wild type PAO1,
103 the clinical strains *P. aeruginosa* Ps4 and Ps74, and the PAO1 derivative PAΔD,
104 an AmpC overexpressing mutant [6]. We also used two ESBL-expressing
105 *Enterobacteriaceae, E. coli* E20 and *K. pneumoniae* K2 (Table 1). For routine
106 procedures, bacteria were grown at 37ºC in Tryptic Soy Broth (TSB; BioMerieux)
107 or in TSB supplemented with 16 g/L agar (TSA; Pronadisa, Spain). TSB was also
108 used as medium for biofilm growth. MICs of antimicrobials were determined in
109 Mueller-Hinton cation adjusted (MHCA) broth (Difco Laboratories) using serial
110 two-fold dilutions according to CLSI guidelines [26], as detailed previously [27].
111 Antimicrobials with MICs higher than the maximum concentration tested were
112 assigned a MIC twice that concentration. MHCA broth was also used for synergy
113 testing (checkerboard assay), growth inhibition experiments (see 1.6) and to test
114 the bactericidal effect of the antimicrobials on biofilm cells.

115 **2.2. Antimicrobial agents**

116 Amoxicillin, Ampicillin, Aztreonam, Ceftazidime, Clavulanic acid, Colistin,
117 Oxacillin, Phenylboronic acid (PBA), Piperacillin, polymyxin B nonapeptide
118 (PMBN), polymyxin B (PMB), Sulbactam, Tazobactam and Ticarcillin, were
119 purchased from Sigma-Aldrich. P4-1, P4-5, P5-3, P5-5, P5-8, P5-9, P5-11, P5-
120 12, P5-17 and P5-19 peptides were synthesized by *PolyPeptide Laboratories*

121 (France). All of them were purified by RP-HPLC (96 % of purity, at least), and
122 their amino acid composition and sequence was confirmed by HPLC and mass
123 spectrometry analysis, respectively. Stock solutions at 10 mg/mL were
124 dissolved as detailed in Supplementary Table 1, according to manufacturer's
125 recommendations. Then, they were diluted in water and then during the synergy
126 testing, they were further diluted in MHCA.

127 **2.3. Extended spectrum β-lactamase (ESBL) phenotypic detection**

128 Two different phenotypic methods were used to detect ESBLs: double disk
129 diffusion test, and combined disk test (Supplementary Material 1.1).

130 **2.4. Real Time quantitative PCR (RT q-PCR)**

131 The expression of the gene *ampC* was quantified by RT q-PCR according to
132 previously described protocols [28] and following manufacturer's
133 recommendations (Invitrogen). Expression was referred to the housekeeping
134 gene *proC* and the reference strain PAO1. The primers used for *ampC*
135 amplification were forward 5'-GGCGACATGACAGGGCCT-3' and reverse 3'-
136 TCCAGGCCGCTGAGGATGGC-5' with a product size of 296 bp and for *proC*
137 amplification, forward 5'-CAGGCCGGGCAGTTGCTGTC-3' and reverse 5'-
138 GGTCAGGCGCGAGGCTGTCT-3' with a product size of 188 bp. A relative
139 expression greater than or equal to 10 was considered overexpression, as
140 described by Cabot et al [28]. The experiment was repeated independently at
141 least three times.

142 **2.5. Synergy testing by checkerboard and Bioscreen**

143 Potential synergistic interactions between two antimicrobials were first assessed
144 by the checkerboard assay using MHCA broth as described before [29]. To
145 quantify synergistic interactions between three antimicrobials a three-
146 dimensional checkerboard test was used [25]. For the latter method, the
147 checkerboard assay was performed in the presence of a fixed concentration of
148 one of the antimicrobials.

149 Kinetics of inhibition of planktonic bacteria by selected antimicrobial combinations
150 were measured in the automated optical analyzer Bioscreen C (Labsystems
151 Laboratories, Helsinki, Finland) in MHCA broth as described before (Ferrer-
152 Espada et al., 2019, Supplementary Material 1.2). Each experiment was
153 independently repeated three times and each concentration was tested in three
154 wells. The inhibitory activity of different treatments was compared by determining
155 the corresponding area under the curve (AUC) during the first 45 h of incubation
156 and by applying the Mann Whitney U Test complemented with Kruskal Wallis
157 comparisons (*, $p<0.05$).

158 **2.6. Biofilm formation and assessment of anti-biofilm activity**

159 Biofilms of *P. aeruginosa* Ps4 were grown under dynamic shear conditions using
160 the CDC-reactor (model CBR 90–1, BioSurface Technologies Corporation,
161 Bozeman, MT. USA) as described elsewhere [30], (Supplementary material 1.3).
162 Mortality due to the treatments was assessed by colony counting and by confocal
163 laser microscopy using the LIVE/DEAD BacLight kit (Life Technologies), as
164 previously reported [30].

165 For the former technique, experiments were independently repeated twice in
166 duplicate coupons and differences between treated and not treated coupons

167 were analyzed by one-way ANOVA followed by Tukey's multiple comparison test
168 (** p<0.01, *** p<0.001).

169 **2.7. Time-kill curve and 1-*N*-phenylnaphthyl-amine (NPN) uptake assays**

170 Kinetics of mortality of planktonic cultures exposed to increasing concentrations
171 of antimicrobials were measured as previously described [30]. Experiments were
172 repeated twice independently.

173 NPN uptake assays were carried out as previously reported by us [29];
174 (Supplementary material 1.4). Values were the average of five independent
175 experiments performed in quadruplicate and results were expressed as relative
176 fluorescence units.

177 **3. RESULTS**

178 **3.1. Strain characterization**

179 The profile of susceptibility of the strains to β-lactams and to other relevant
180 antimicrobials is shown in Table 2. The wild type levels of AmpC expressed by
181 PAO1 conferred this strain non-susceptibility to amoxicillin and ampicillin but not
182 to the rest of β-lactams tested. In contrast, the clinical isolate Ps4 was uniformly
183 resistant to all the β-lactams, whereas PAΔD expressed a pattern similar to Ps4,
184 being only susceptible to ticarcillin and aztreonam. Interestingly, this profile was
185 in good correlation with the levels of *ampC* expressed by each strain, because
186 Ps4 produced levels of the *ampC* transcript much higher than those of PAΔD
187 (Fig. S1).

188 On the other hand, results of the double disk diffusion tests with PAΔD and Ps4
189 indicated that these two strains do not express β-lactamases susceptible to

190 inhibition by clavulanic acid, such as ESBL (Fig. S2). In contrast, the susceptibility
191 pattern of the two *Enterobacteriaceae* (Table 2), their clavulanic acid dependent
192 sensitization to β-lactams (Fig. S2 and Fig. S3) and additional phenotypic testing
193 (not shown) strongly suggest that they are ESBL producers.

194 **3.2. PMBN sensitizes ESBL-expressing *Enterobacteriaceae* to BLI**

195 Synergy testing of *Enterobacteriaceae* strains by checkerboard confirmed that
196 classic inhibitors of ESBL, such as sulbactam, tazobactam and clavulanic acid,
197 enhanced susceptibility to β-lactams in these strains, thus supporting their
198 consideration as ESBL-expressing organisms (Table 3; first row of panels A and
199 B). However, in all cases such enhancement was clinically irrelevant, since
200 addition of the inhibitor brought down MICs to values still far from the threshold
201 of susceptibility. For instance, the most potent BLI, tazobactam, reduced *E. coli*
202 E20 MIC to piperacillin from 256 mg/L to 32 mg/L, a value 2 times higher than
203 that considered as the susceptibility breakpoint (16 mg/L; EUCAST, 2019).
204 Interestingly, when this same assay was performed in the presence of very low
205 concentrations of PMBN (2 mg/L) a full sensitization to piperacillin was achieved
206 (Table 3).

207 Results shown in Table 3 demonstrate that PMBN-mediated enhancement of
208 antibiotic activity occurs in a dose dependent manner, not only with piperacillin
209 but also with all the β-lactams tested. Importantly, this enhancement was strictly
210 dependent on β-lactamase inhibitory activity, since addition of PMBN had a minor
211 effect (or it was null) in the absence of the BLI. Sensitization of *E. coli* E20 to
212 other antibiotics such as ticarcillin (breakpoint value= 16 mg/L) occurred at the
213 highest concentrations of PMBN tested (8 and 16 mg/L), likely reflecting the high

214 basal level of resistance to this antibiotic displayed by the organism (>512 mg/L).
215 On the other hand, PMBN-mediated enhancement was not potent enough to
216 lower *E. coli* E20 MICs to ampicillin-sulbactam or to amoxicillin-clavulanic acid
217 below the susceptibility breakpoints (i.e. 8 mg/L for both combinations).

218 Despite the very high level of resistance to β-lactams exhibited by *Klebsiella*
219 *pneumoniae* K2, addition of 16 mg/L of PMBN strongly sensitized it to all the
220 antibiotic-BLI combinations tested. In all the cases, the lowest MIC value
221 achieved was 16 mg/L, which is only one dilution above the susceptibility
222 breakpoint for amoxicillin and ampicillin and makes it susceptible to piperacillin
223 and ticarcillin. In agreement with these results, the parameter used to quantify
224 antibiotic-enhancing activity, FICI, indicated that the majority of three-component
225 combinations (Table 3; values in bold) were synergistic (i.e. FICI≤0.5).

226 To confirm and expand these results, we used a turbidimetric method (Bioscreen
227 C) to measure the kinetics of growth of the test strains in the presence of the most
228 potent three-component combinations. Specifically, *E. coli* CUN E20 and *K.*
229 *pneumoniae* CUN K2 were exposed to piperacillin (2 mg/L)/tazobactam (4
230 mg/L)/PMBN (8 mg/L) and piperacillin (16 mg/L)/tazobactam (2 mg/L)/PMBN (16
231 mg/L), respectively. As shown in Fig. 1, the selected combinations abrogated
232 growth of both organisms for the entire duration of the experiment (48h). In fact,
233 exposure to any double combination or single component adjusted to the
234 concentration present in the triple combination had a null effect on the growth of
235 *E. coli*. In the case of *K. pneumoniae,* the double combination Piperacillin (16
236 mg/L)/Tazobactam (2 mg/L) prevented growth during the first 10 h, although
237 comparison of the respective areas under the curves indicated that this effect was

238 not statistically significant. These results demonstrate that the three compounds
239 had to be present in the combinations to efficiently abolish bacterial growth.

240 ### 3.3. PMBN and other permeability enhancers sensitize *P. aeruginosa*
241 ### PAΔD to BLI

242 For these experiments, we first used the PAΔD mutant strain and BLIs specific of
243 AmpC such as Oxacillin, PBA and Aztreonam. The most interesting results were
244 obtained with Aztreonam whose inhibitory activity against PAΔD was potentiated
245 several orders of magnitude by PMBN (Table 4). Specifically, addition of 2 mg/L
246 of PMBN reduced the MIC of ceftazidime from 16 mg/L to ≤0.03 mg/L in the
247 presence of 1 mg/L of Aztreonam. Table 4 also shows that the presence of this
248 BLI was necessary for the full enhancing effect, since a duplicate experiment
249 performed in the absence of Aztreonam rendered a much more modest
250 sensitization (from 16 mg/L to 4 mg/L). Similar results, although not as potent as
251 those observed with Aztreonam, were obtained with PBA and Oxacillin.

252 To investigate whether AMPs other than PMBN possess BLI-potentiating activity
253 we repeated the previous assays using human lactoferricin-derived peptides
254 designed by us. Similar to PMBN, these compounds have both poor antimicrobial
255 activity against PAΔD (see MICs in Table 5) and potent permeabilizing capacity
256 (unpublished results). As shown in Table 5, at concentrations as low as 6.25 mg/L
257 peptides P5-8 and P5-9 caused a very potent sensitization to ceftazidime in the
258 presence of PBA. In control experiments, the peptides failed to enhance antibiotic
259 activity in the absence of the BLI. Other peptides displayed a similar behavior,
260 although at higher concentration than P5-8 and P5-9.

261 ### 3.4. PMBN sensitizes a multi-drug resistant *P. aeruginosa* clinical strain
262 Ps4 to BLI

263 In addition to overexpressing AmpC, Ps4 hyper-produces the MexAB-OprM efflux
264 pump system [25]. Notably, this efflux pump is capable of expelling BLIs such as
265 Aztreonam and Cloxacillin [31,32], thus making Ps4 a challenging target for our
266 combinatorial strategy. When added at 4 mg/L, PMBN had a significant
267 ceftazidime enhancing activity in the absence of BLI, being able to reduce the
268 antibiotic MIC from 64 to 4 mg/L (Table 6). Interestingly, when the assay was
269 repeated in the presence of 1 mg/L of Aztreonam, a much more potent
270 sensitization was detected and MIC value dropped down to ≤0.03 mg/L. Similar
271 results were obtained with PBA, although this BLI showed less potency. For
272 unknown reasons, at high PMBN concentrations (16 mg/L) the nonapeptide
273 partially lost its ceftazidime enhancing activity both alone and when combined
274 with PBA but not with Aztreonam.

275 The kinetic assays (Fig. 2) revealed that the nonapeptide alone was able to
276 abolish growth during the first 13h of growth at the selected concentration (4
277 mg/L). Under these conditions, addition of Ceftazidime and/or Aztreonam did not
278 cause a significant growth delay as assessed by the analysis of the area under
279 the curve. In contrast, when PBA was used as component of the triple
280 combination, the inhibitory effect was significantly stronger than any other
281 treatment.

282 ### 3.5. Assessment of the permeability-increasing potency of the
283 combination components

284 As shown in Fig. 3, neither the antibiotic nor the BLI showed permeabilizing
285 activity at the concentrations used in previous assays (Fig. 2). In contrast, NPN
286 rapidly incorporated into PMBN-treated Ps4 cells with kinetics indistinguishable
287 from those of cells exposed to the triple combination. Consistent with our
288 hypothesis on the mechanism of action of PMBN-mediated enhancement, a
289 colistin resistant strain of *P. aeruginosa* (Ps74) was totally non-susceptible to the
290 triple combination (Table S2).

291 **3.6. Evaluation of the anti-biofilm activity of the triple combination**

292 To study whether the Ceftazidime-PBA-PMBN combination was inhibitory or
293 bactericidal, we first determined the time-kill curve of planktonically grown Ps4
294 exposed to this combination. Specifically, the triple combination was tested at the
295 following concentrations: 1 x MIC (equivalent to 0.06 mg/L of Ceftazidime-2 mg/L
296 of PBA-4 mg/L of PMBN), 2 x MIC and 4 x MIC, and samples were taken
297 periodically for viable counting. Fig. S4 shows that at 1 x MIC the combination
298 completely inhibited bacterial growth during the entire duration of the assay (6h).
299 However, at concentrations higher than its MIC, the treatment was bactericidal in
300 a dose-dependent manner. Thus, the concentration 4 x MIC reduced the bacterial
301 viability of the initial inoculum approximately 100 times (from $10^6$ to $10^4$ CFU/mL)
302 in 6h.

303 In addition, the combination Ceftazidime-PBA-PMBN was tested at 50 x MIC on
304 mature biofilms grown under turbulent flow in the CDC-reactor. As shown in Fig.
305 4 B, the treatment reduced biofilm viability $10^8$ times in 72 h, thus showing a
306 bactericidal efficacy comparable to that of the control treatment (sodium
307 hypochlorite). It is worth to note that a standard treatment used for antibiotic lock

308 therapy (Ceftazidime 5000 mg/L) was unable to reduce the viability of the biofilm
309 in the same conditions (data not shown, [25]). These assays also revealed that
310 the presence of the PBA did not improve significantly the anti-biofilm efficacy of
311 the double combination 50 x Ceftazidime/PMBN. In contrast, the elimination of
312 either the antibiotic or the peptide from the triple combination reduced 5-6 orders
313 of magnitude its bactericidal activity. Notably, PMB or PME (Colistin) at 50 times
314 their planktonic MIC showed an anti-biofilm activity similar to that of the triple
315 combination.

316 Finally, in independent assays the ability of the experimental treatment to kill pre-
317 formed biofilm was also studied by confocal laser microscopy using fluorescent
318 probes. As shown in Fig. 4, untreated biofilms stained green, indicating that their
319 cells were alive, whereas duplicate biofilms treated with either hypochlorite or
320 with the combination 50 x Ceftazidime-PBA-PMBN for 24 h in MHCA, stained red,
321 thus confirming that these two treatments were bactericidal.

## 4. DISCUSSION

This is the first demonstration that BLIs can be enhanced by a permeability-increasing agent added at subinhibitory concentrations. We showed that this strategy can be used to neutralize β-lactamase activity, thus sensitizing the β-lactamase-producing organism to beta-lactams. Interestingly, our results suggest that this type of potentiation may have a broad spectrum of activity, since it counteracted two unrelated classes of β-lactamases, ESBLs (class A) and AmpC (class C), in *Enterobacteriaceae* and *Pseudomonas*, respectively.

Taken together, our data strongly suggest that the mechanism of sensitization depends on blockade of β-lactamase activity caused by the permeabilizing effect of the peptide. On the one hand, PMBN is a well-known permeability enhancer [33] and our experiments with NPN showed that only the peptide had permeabilizing capacity. This property is also very prominent in the case of P5 peptides (Table 5~~our unpublished results~~), which were also able to potentiate BLIs. Importantly, the inability of PMBN to enhance BLI when tested on the *P. aeruginosa* strain resistant to colistin (Ps74) confirms that peptide dependent permeabilizing activity is essential for sensitization.

On the other hand, although the peptide often acted in synergy with the antibiotic (i.e. two-component combination; Table 4), the BLI was strictly required to achieve full enhancing capacity. This demonstrates that the β-lactamase remained susceptible to the inhibitor and suggests that the mechanism of sensitization depended on enzyme blockade, probably caused by its saturation due to the massive entry of BLI into the periplasm. In the absence of the peptide,

345 it is very likely that levels of inhibitor inside the periplasm would not be high
346 enough to neutralize the activity of the β-lactamase.

347 In addition to enhancing BLI activity, peptide-mediated permeabilization is
348 expected to lead to accumulation of the antibiotic inside the periplasm. Since the
349 β-lactam binding sites (i.e. PBPs), are preferentially located in the periplasm, this
350 would further bolster antimicrobial activity and explain the potent sensitization
351 shown in this work. In agreement with the proposed mechanism, we have recently
352 demonstrated that efflux pump inhibitors, whose targets are located in the
353 periplasm, can also be enhanced by PMBN, thus sensitizing bacteria to
354 antibiotics substrate of efflux pumps [25].

355 Our observations could explain synergism reported by other authors when
356 antimicrobial peptides were combined with BLI and β-lactams against Gram-
357 negative bacteria. Thus, Ghiselli and collaborators reported that combinations of
358 indolicidin/piperacillin/tazobactam possess *in vitro* synergistic activity against *E.*
359 *coli* and *E. faecalis* [34]. However, they did not determine the actual contribution
360 of the BLI to the overall efficacy of the combined treatment, since controls with
361 the β-lactam alone (without the inhibitor) were not included.

362 Similarly, other researchers reported synergism between colistin and
363 ampicillin/sulbactam, although they used concentrations of colistin higher than its
364 MIC, while in the present work the permeabilizing agents were added at sub-MIC
365 levels [35]. Finally, Mikhail *et al.* tested a strategy similar to ours with the
366 combination ceftazidime/avibactam/colistin but failed to report synergy likely due
367 to the poor antibiotic enhancing activity of colistin compared with PMBN [36]. In

368 this respect, it is important to note that good permeabilizers do not necessarily
369 correspond to compounds with potent bactericidal activity [27,29].

370 Our results also demonstrate that, at 50 times its planktonic MIC, the
371 experimental triple combination was as active against mature biofilms as the most
372 lethal treatment tested (i.e. chlorine). This suggests that the combination could
373 be applied in antibiotic lock therapy (ALT), a treatment used to eliminate biofilms
374 formed in the lumen of medical devices implanted in patients. This is more so
375 considering that the combination was applied for 72 h, whereas solutions for ALT
376 are normally allowed to act for at least 10 days [37] at 1000 x MIC. Nevertheless,
377 the fact that the PMB based monotherapy matched the anti-biofilm activity of the
378 peptide/BLI/β-lactam triple combination reduces the attractiveness of the latter
379 for use in ALT.

380 The full inhibition of growth achieved with the combined treatment against *E. coli*
381 and *K. pneumoniae* (Fig. 1) suggests that this therapy could also be effective *in*
382 *vivo*. In this context, other authors showed that PMBN retains its antibiotic-
383 enhancing capacity *in vivo* in murine models of experimental infection [38,39].
384 The low toxicity of PMBN compared with that of its parental compound, PMB
385 (LD50: 43 vs. 8 mg/kg-mouse, respectively) [39] should result in a better
386 therapeutic index of the nonapeptide with respect to the native molecule. Indeed,
387 a molecule very similar to PMBN, the compound SPR741/NAB741, recently
388 passed clinical phase 1 trials [40].

389 **CONCLUSION**

390 Our results support the hypothesis that AMPs can synergize with BLIs and that
391 this phenomenon can be exploited to sensitize bacteria to antibiotics. However,

392 *in vivo* experimentation is indispensible to study the potential therapeutic
393 applicability of our results.”

394

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**Table 1.- Relevant characteristics of the bacterial strains used in this work**

| Strain | Relevant features | Source or reference |
|---|---|---|
| *Pseudomonas aeruginosa* | | |
| PAO1 | Wild Type. | CECT[1] 4122 |
| PAΔD | PAO1 derivative carrying a mutation in *ampD* that causes the overexpression of the AmpC β-lactamase (Supplementary Figs. 1 and 2). | [28] |
| Ps4 | AmpC β-lactamase and MexAB-OprM overexpressing strain isolated from sputum at CUN (Supplementary Figs. 1 and 2). | [25] |
| Ps74 | AmpC β-lactamase overexpressing strain isolated from sputum at CUN (Supplementary Fig. 1). Colistin resistant. | [27] |
| *Escherichia coli* E20 | Extended-spectrum β-lactamase expressing strain isolated from urethral swab (Supplementary Figs. 2 and 3). | CUN |
| *Klebsiella pneumoniae* K2 | Extended-spectrum β-lactamase expressing strain isolated from perianal swab (Supplementary Fig. 3) | CUN |

[1]: Spanish Type Culture Collection, [2]: Clínica Universidad de Navarra (CUN; University Hospital of Navarra).

**Table 2.- Antimicrobial susceptibility of the strains used in this work.**

| Antimicrobials | | | MIC[1] (mg/L) | | | | |
|---|---|---|---|---|---|---|---|
| | | | *Pseudomonas aeruginosa* | | | *Escherichia coli* | *Klebsiella pneumoniae* |
| | | | PAO1 | PAΔD | Ps4 | E20 | K2 |
| β-lactams | Penicillins | Amoxicillin | 2048 | >512 | >512 | >512 (R[2]) | >512 (R) |
| | | Ampicillin | 1024 | >512 | >512 | >512 (R) | >512 (R) |
| | | Piperacillin | 4 (S[3]) | 128 (R) | 256 (R) | 256 (R) | >512 (R) |
| | | Ticarcillin | 16 (S) | 64 (S) | 256 (R) | >512 (R) | >512 (R) |
| | Cephalosporins | Ceftazidime | 2 (S) | 16 (I[4]) | 64 (R) | nd[5] | nd |
| AMPs[6] | Colistin | | 1 (S) | 2 (S) | 1 (S) | 0.25 | 0.25 |
| | PMB[7] | | 1 (S) | nd | 1 (S) | 0.25 | 0.25 |
| | PMBN[8] | | >512 | >512 | >512 | >512 | 512 |
| Inhibitors | AmpC | PBA[9] | >512 | >512 | >512 | nd | nd |
| | | Aztreonam | 4 (S) | 8 (S) | 16 (I) | nd | nd |
| | | Oxacillin | >512 | >512 | >512 | nd | nd |
| | ESBL[10] | Sulbactam | >512 | >512 | >512 | 32 | 64 |
| | | Tazobactam | nd | >512 | nd | 512 | 256 |
| | | Clavulanic acid | 128 | 128 | 256 | 32 | 32 |

[1]: Minimum inhibitory concentration; [2]: Resistant; [3]: Susceptible; [4]: Intermediate according to Clinical and Laboratory Standards Institute (CLSI) guidelines; [5]: not determined; [6]: Antimicrobial peptides; [7]: Polymyxin B; [8]: Polymyxin B Nonapeptide. [9]: Phenylboronic acid. [10]: Extended-spectrum β-lactamase.

**Table 3.- PMBN enhances β-lactamase inhibitors and sensitizes two ESBL-expressing *Enterobacteriaceae* to β-lactam antibiotics, as assessed by checkerboard testing.**

(A) *Escherichia coli* E20

| PMBN (mg/L) | MIC[1] of piperacillin (mg/L) in the presence of the concentration of inhibitor indicated | | FICI[2] | MIC of ticarcillin (mg/L) in the presence of the concentration of inhibitor indicated | | FICI | MIC of amoxicillin (mg/L) in the presence of the concentration of inhibitor indicated | | FICI | MIC of ampicillin (mg/L) in the presence of the concentration of inhibitor indicated | | FICI |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | Tazobactam (4 mg/L) | | 0 | Clavulanic acid (4 mg/L) | | 0 | Clavulanic acid (4 mg/L) | | 0 | Sulbactam (4 mg/L) | |
| 0 | 256 | 32 | | >512 | 128 | | >512 | 128 | | >512 | 256 | |
| 1 | 128 | 32 | **0.13** | >512 | 128 | **0.25** | >512 | 128 | **0.25** | >512 | >256 | 0.63 |
| 2 | 64 | 8 | **0.04** | >512 | 128 | **0.25** | >512 | 64 | **0.19** | >512 | 256 | **0.38** |
| 4 | 64 | 4 | **0.03** | >512 | 32 | **0.16** | >512 | 64 | **0.19** | >512 | 128 | **0.25** |
| 8 | 64 | 2 | **0.02** | >512 | 16 | **0.15** | 512 | 32 | **0.16** | >512 | 128 | **0.26** |
| 16 | 64 | 1 | **0.03** | 512 | 8 | **0.15** | 512 | 16 | **0.16** | >512 | 64 | **0.20** |

(B) *Klebsiella pneumoniae* K2

| PMBN (mg/L) | MIC[1] of piperacillin (mg/L) in the presence of the concentration of inhibitor indicated | | FICI | MIC of ticarcillin (mg/L) in the presence of the concentration of inhibitor indicated | | FICI | MIC of amoxicillin (mg/L) in the presence of the concentration of inhibitor indicated | | FICI | MIC of ampicillin (mg/L) in the presence of the concentration of inhibitor indicated | | FICI |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | Tazobactam (2 mg/L) | | 0 | Clavulanic acid (4 mg/L) | | 0 | Clavulanic acid (4 mg/L) | | 0 | Sulbactam (16 mg/L) | |
| 0 | >512 | 512 | | >512 | 512 | | >512 | 128 | | >512 | >512 | |
| 1 | >512 | 512 | 0.51 | >512 | 64 | **0.19** | >512 | 64 | **0.19** | >512 | 512 | 0.75 |
| 2 | >512 | 512 | 0.51 | >512 | 64 | **0.19** | >512 | 64 | **0.19** | >512 | 256 | **0.50** |
| 4 | >512 | 256 | **0.27** | >512 | 64 | **0.20** | >512 | 64 | **0.20** | >512 | 128 | **0.38** |
| 8 | >512 | 256 | **0.27** | >512 | 32 | **0.17** | >512 | 32 | **0.17** | >512 | 64 | **0.33** |
| 16 | >512 | 16 | **0.05** | >512 | 16 | **0.17** | >512 | 16 | **0.17** | >512 | 16 | **0.30** |

[1]: Minimum inhibitory concentration. [2]: Fractional inhibitory concentration index. Synergistic combinations (FICI<0.5) are indicated in bold.

**Table 4.-** PMBN enhances β-lactamase inhibitors and sensitizes *Pseudomonas aeruginosa* PAΔD to ceftazidime.

| PMBN (mg/L) | MIC[1] of ceftazidime (mg/L) in the presence of the concentration of inhibitor indicated | | | | | | |
|---|---|---|---|---|---|---|---|
| | 0 | Aztreonam (1 mg/L) | FICI[2] | PBA (2 mg/L) | FICI | Oxacillin (4 mg/L) | FICI |
| 0 | 16 | 16 | | 2 | | 8 | |
| 1 | 16 | 16 | 1.13 | 2 | **0.13** | 16 | 1.00 |
| 2 | 4 | ≤0,03 | **0.13** | 0.06 | **0.01** | 2 | **0.13** |
| 4 | 4 | ≤0,03 | **0.13** | ≤0,03 | **0.01** | 0.25 | **0.02** |
| 8 | 4 | ≤0,03 | **0.13** | 0.06 | **0.01** | 0.25 | **0.03** |
| 16 | 2 | ≤0,03 | **0.14** | 0.12 | **0.03** | 0.5 | **0.05** |

[1]: Minimum inhibitory concentration. [2]: Fractional inhibitory concentration index Synergistic combinations (FICI<0.5) are indicated in bold.

**Table 5.- Human lactoferricin derived peptides enhance PBA activity and sensitize *Pseudomonas aeruginosa* PAΔD to ceftazidime.**

| Peptide (MIC mg/L) | Sequence | MIC[1] of ceftazidime (mg/L) in the presence of 2 mg/L of PBA[2] and the concentrations of peptide (mg/L) indicated | | | | | | FICI[2] min |
|---|---|---|---|---|---|---|---|---|
| | | 0 | 1.5 | 3.125 | 6.25 | 12.5 | 25 | |
| P4-1 (>512) | PFWRRFWRRR-$NH_2$ | 4 | 8 | 8 | 8 | 4 | 4 | **0.26** |
| P4-5 (128) | RRRIWRRWFI-$NH_2$ | 4 | 4 | 4 | 4 | ≤0,03 | ≤0,03 | **0.10** |
| P5-3 (64) | WRRWRRWRRWRR-$NH_2$ | 4 | 8 | 4 | 4 | ≤0,03 | ≤0,03 | **0.20** |
| P5-5 (>512) | FWRRNFWRRNIRR-$NH_2$ | 4 | 8 | 8 | 4 | ≤0,03 | ≤0,03 | **0.02** |
| P5-8 (256) | PFWRIRWRR-$NH_2$ | 4 | 8 | 4 | ≤0,03 | ≤0,03 | ≤0,03 | **0.03** |
| P5-9 (256) | FWRWRRWIRR-$NH_2$ | 4 | 4 | 4 | ≤0,03 | ≤0,03 | ≤0,03 | **0.03** |
| P5-11 (>512) | PFWRRWRR-$NH_2$ | 4 | 8 | 8 | 8 | 8 | 4 | **0.28** |
| P5-12 (64) | WFRRIWRRIRR-$NH_2$ | 4 | 2 | 2 | 2 | 2 | 2 | **0.15** |
| P5-17 (>512) | FWRRIWRR-$NH_2$ | 4 | 2 | 2 | 4 | 4 | 2 | **0.13** |
| P5-19 (512) | RRPFWRRPFWRR-$NH_2$ | 4 | 4 | 4 | 4 | ≤0,03 | ≤0,03 | **0.03** |

[1]: Minimum inhibitory concentration. [2] Phenylboronic Acid. The lowest MIC value obtained in the absence of PBA (i.e. peptide-ceftazidime combinations) was 0.25 mg/L using 25 mg/L of peptide, whereas at lower peptide concentrations this value was always ≥1 [2]: Fractional inhibitory concentration index. Synergistic combinations are indicated in bold

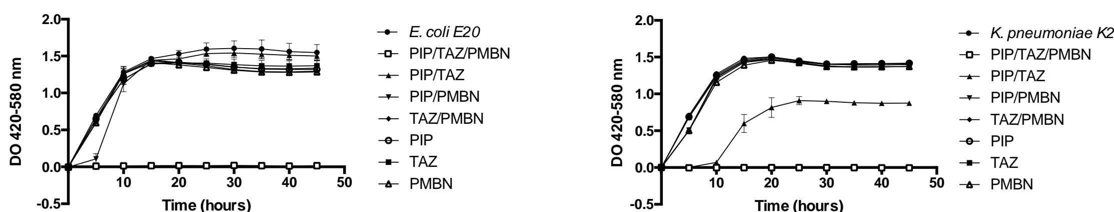
**Table 6.- PMBN enhances β-lactamase inhibitors activity and sensitizes *Pseudomonas aeruginosa* Ps4 to ceftazidime.**

| PMBN (mg/L) | MIC[1] of ceftazidime (mg/L) in the presence of the concentration of inhibitor indicated | | | | |
|---|---|---|---|---|---|
| | 0 | Aztreonam (1 mg/L) | FICI[2] | PBA (2 mg/L) | FICI[2] |
| 0 | 64 | >16 | | 16 | |
| 1 | >16 | >16 | 0.56 | 16 | **0.25** |
| 2 | >16 | >16 | 0.56 | 8 | **0.13** |
| 4 | 4 | ≤0,03 | **0.07** | 0.06 | **0.01** |
| 8 | 0.5 | ≤0,03 | **0.07** | 0.12 | **0.01** |
| 16 | 8 | ≤0,03 | **0.08** | 0.5 | **0.03** |

[1]: Minimum inhibitory concentration. [2]: Fractional inhibitory concentration index.

Synergistic combinations (FICI<0.5) are indicated in bold.

**A:** Growth curve




**B:** Area under the curve

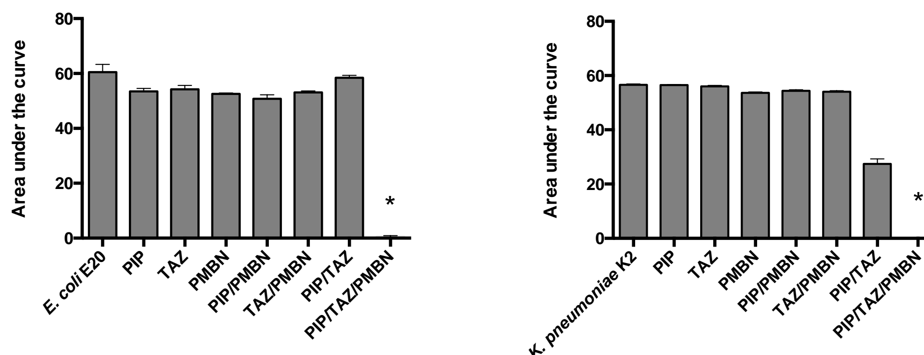



**Figure 1.- PMBN enhances tazobactam and abrogates growth of two ESBL-expressing *Enterobacteriaceae,* as assessed by turbidimetry (Bioscreen C).** (A) Growth kinetics of *Escherichia coli* E20 (left panel) and *Klebsiella pneumoniae* K2 (right panel) in the presence of the indicated antimicrobial combinations. At time 0, cultures were exposed to the indicated antimicrobial combinations and incubated with shaking at 37 ºC in an optical analyzer that automatically monitors optical density at regular intervals. The inoculum and the culture medium (MHCA) were the same as those used for MIC determination. Antimicrobials were added at the following concentrations: left panel; PIP: piperacillin (2 mg/L); TAZ: tazobactam (4 mg/L); PMBN (8 mg/L); right panel; PIP: piperacillin (16 mg/L); TAZ: tazobactam (2 mg/L); PMBN (16 mg/L). (B) Area under the curve during the first 45 h of growth of indicated cultures. Results shown are the means ± standard error of three independent experiments where each concentration was tested in triplicate wells (n=9). Data were analyzed using Kruskal Wallis test with multiple comparisons and statistical differences between the culture treated with the triple combination and the untreated control were significant (*; p=0.0280 for *K. pneumoniae* K2 and p=0.0227 for *E. coli* E20).

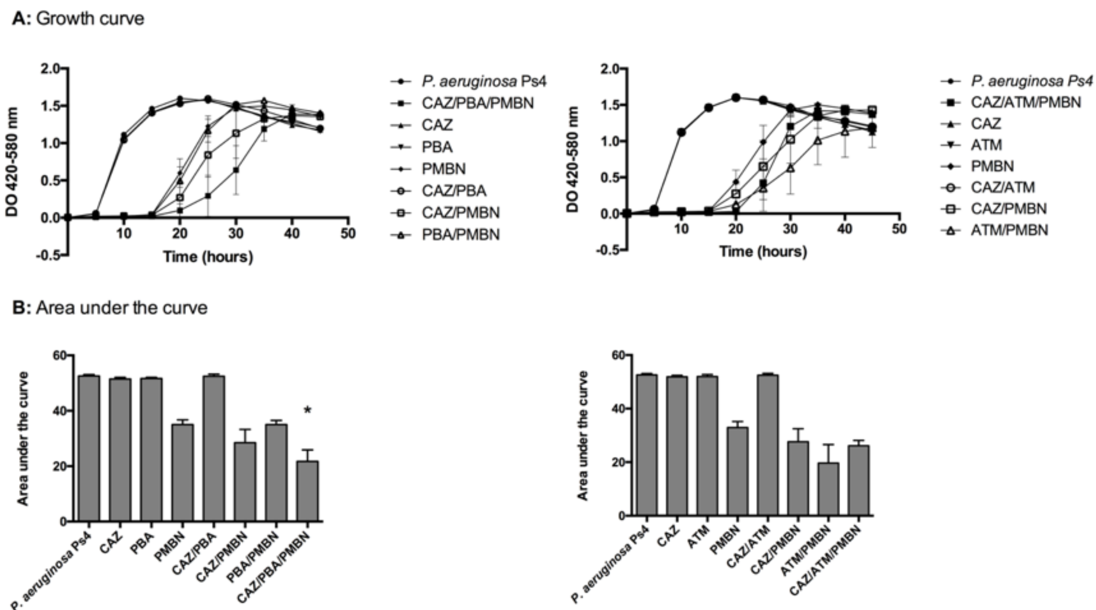
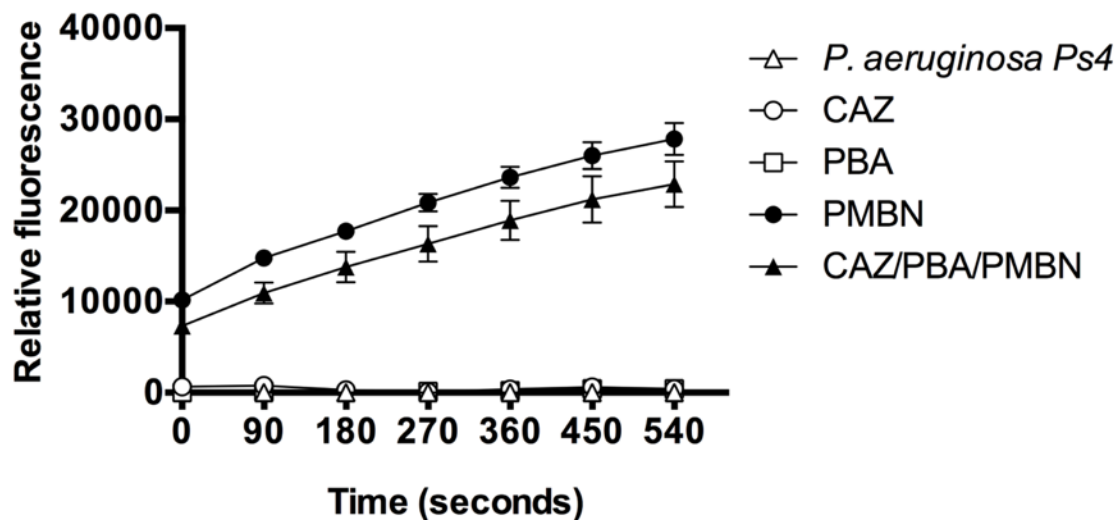


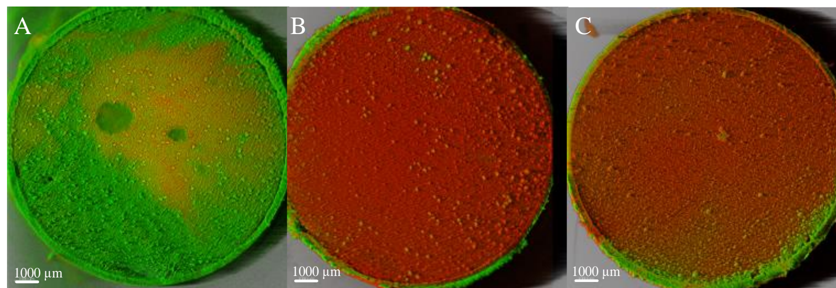

**Figure 2.- PMBN enhances β-lactamase inhibitors (phenylboronic acid -graphs on the left- or aztreonam -graphs on the right-) and sensitizes an AmpC overexpressing *Pseudomonas aeruginosa* clinical strain (Ps4) to ceftazidime, as determined by turbidimetry (Bioscreen C).** (A) at time 0, cultures were exposed to the indicated antimicrobial combinations and incubated with shaking at 37 ºC in an optical analyzer that automatically monitors optical density at regular intervals. The inoculum and the culture medium (MHCA) were the same as those used to determine the MIC. CAZ: ceftazidime (0.03 mg/L left panels, 0.06 mg/L right panels); PBA: phenylboronic acid (2 mg/L); PMBN (4 mg/L); ATM: aztreonam (1 mg/L). (B) Area under the curve during the first 45 h of growth of indicated cultures (panel (A)). Results shown are the means ± standard error of four independent experiments where each concentration was tested in triplicate wells (n=12). Data were analyzed using Kruskal Wallis test with multiple comparisons and statistical differences between the culture treated with the triple combination and the untreated control were significant for the combination with phenylboronic acid (*; p=0.0194).



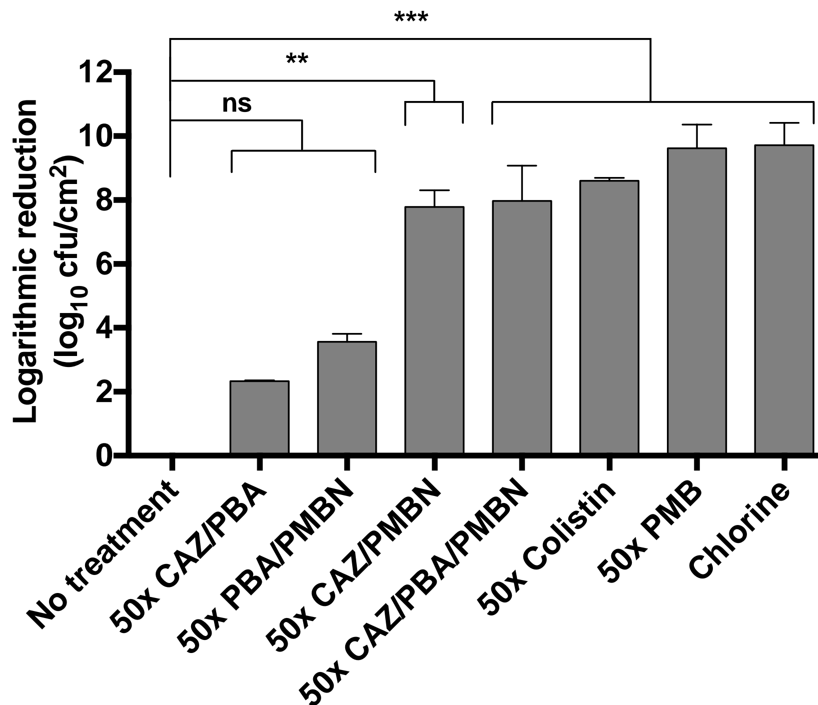

38

39 **Figure 3.- Permeabilizing activity of PMBN combinations containing**
40 **antibiotic and β-lactamase inhibitors is due only to PMBN, as assessed by**
41 **uptake of the fluorescent probe 1-*N*-phenylnaphthyl-amine (NPN).** *P.*
42 *aeruginosa* Ps4 in the exponential phase was resuspended in HEPES pH 7.2
43 supplemented with 0.1% glucose, exposed to the agent under study and after the
44 addition of NPN, the fluorescence was measured in a spectrofluorometer at 37
45 ºC. Compounds were tested either alone or as part of the triple combination at
46 the following concentrations: Polymyxin B Nonapeptide (4 mg/L; PMBN),
47 phenylboronic acid (2 mg/L; PBA), ceftazidime (0.06 mg/L; CAZ). Results shown
48 are the means ± standard error of five independent studies where each
49 measurement was carried out in quadruplicate wells (n=20).

(A) Confocal imaging




(B) Plate counts




50

51 **Figure 4.- Bactericidal activity of antimicrobial combinations against mature**
52 **biofilms of *Pseudomonas aeruginosa* Ps4 grown in the CDC biofilm reactor**
53 **for 48h.** After 48 h of growth under turbulent conditions, mature biofilms were
54 removed from the reactor, washed and exposed for 24 h (panel A) or 72 h (panel B);
55 with renewal of the solution every 24 h to either a positive control of bactericidal
56 activity (chlorine 1,000 mg/L) or to the indicated antimicrobial combinations (A) the
57 surface of the coupons was stained with the commercial kit LIVE/DEAD and
58 visualized with confocal laser microscopy at 1.5X. Scale bars= 1000 µm. A.
59 Viability control, untreated biofilm, B. Bactericidal efficacy control: Chlorine (1000
60 mg/L) and C. Biofilm treated with the triple combination at 50 times their
61 planktonic MIC (Final concentration: CAZ: ceftazidime (3 mg/L); PMBN (200
62 mg/L); PBA: Phenylboronic acid (100 mg/L)). (B) For the plate counts, after 72h of

63 exposure to the treatment at 50 times their planktonic MIC (Final concentration:
64 CAZ: ceftazidime (3 mg/L); PMBN (200 mg/L); PBA: Phenylboronic acid (100
65 mg/L); PMB: Polymyxin B (50 mg/L); Colistin: (50 mg/L)), biofilms were detached
66 from the coupons by scraping, homogenized by sonication and viable bacteria were
67 enumerated. Finally, the logarithmic reduction of viable cells caused by each
68 treatment was calculated using untreated coupons as reference. Results shown are
69 the means $\pm$ standard error of two independent experiments where each condition
70 was tested in duplicate coupons (n=4). Differences between the untreated biofilm
71 and the treated biofilms were analyzed with a One-Way-ANOVA followed by
72 Tukey's multiple comparison test. Differences were not significant (ns; p>0.05),
73 very significant (**; p<0.01), or highly significant (***; p<0.001).

| Tukey's multiple comparisons test | Mean Diff. | 95.00% CI of diff. | Significant? | Summary | Adjusted P Value |
|---|---|---|---|---|---|
| Chlorine vs. 50x CAZ/PBA/PMBN | 1.742 | -1.647 to 5.13 | No | ns | 0.6218 |
| Chlorine vs. 50x CAZ/PBA | 7.377 | 3.091 to 11.66 | Yes | *** | 0.0006 |
| Chlorine vs. 50x CAZ/PMBN | 1.937 | -2.349 to 6.222 | No | ns | 0.7465 |
| Chlorine vs. 50x PBA/PMBN | 6.157 | 1.871 to 10.44 | Yes | ** | 0.0032 |
| Chlorine vs. No treatment | 9.717 | 5.431 to 14 | Yes | **** | <0.0001 |
| Chlorine vs. 50x PMB | 0.09167 | -4.194 to 4.377 | No | ns | >0.9999 |
| Chlorine vs. 50x Colistin | 1.117 | -3.169 to 5.402 | No | ns | 0.9788 |
| 50x CAZ/PBA/PMBN vs. 50x CAZ/PBA | 5.635 | 1.089 to 10.18 | Yes | * | 0.0110 |
| 50x CAZ/PBA/PMBN vs. 50x CAZ/PMBN | 0.195 | -4.351 to 4.741 | No | ns | >0.9999 |
| 50x CAZ/PBA/PMBN vs. 50x PBA/PMBN | 4.415 | -0.1308 to 8.961 | No | ns | 0.0598 |
| 50x CAZ/PBA/PMBN vs. No treatment | 7.975 | 3.429 to 12.52 | Yes | *** | 0.0005 |
| 50x CAZ/PBA/PMBN vs. 50x PMB | -1.65 | -6.196 to 2.896 | No | ns | 0.8917 |
| 50x CAZ/PBA/PMBN vs. 50x Colistin | -0.625 | -5.171 to 3.921 | No | ns | 0.9996 |
| 50x CAZ/PBA vs. 50x CAZ/PMBN | -5.44 | -10.69 to -0.191 | Yes | * | 0.0398 |
| 50x CAZ/PBA vs. 50x PBA/PMBN | -1.22 | -6.469 to 4.029 | No | ns | 0.9888 |
| 50x CAZ/PBA vs. No treatment | 2.34 | -2.909 to 7.589 | No | ns | 0.7580 |
| 50x CAZ/PBA vs. 50x PMB | -7.285 | -12.53 to -2.036 | Yes | ** | 0.0043 |
| 50x CAZ/PBA vs. 50x Colistin | -6.26 | -11.51 to -1.011 | Yes | * | 0.0148 |
| 50x CAZ/PMBN vs. 50x PBA/PMBN | 4.22 | -1.029 to 9.469 | No | ns | 0.1616 |
| 50x CAZ/PMBN vs. No treatment | 7.78 | 2.531 to 13.03 | Yes | ** | 0.0024 |
| 50x CAZ/PMBN vs. 50x PMB | -1.845 | -7.094 to 3.404 | No | ns | 0.9060 |
| 50x CAZ/PMBN vs. 50x Colistin | -0.82 | -6.069 to 4.429 | No | ns | 0.9990 |
| 50x PBA/PMBN vs. No treatment | 3.56 | -1.689 to 8.809 | No | ns | 0.3144 |
| 50x PBA/PMBN vs. 50x PMB | -6.065 | -11.31 to -0.816 | Yes | * | 0.0187 |
| 50x PBA/PMBN vs. 50x Colistin | -5.04 | -10.29 to 0.209 | No | ns | 0.0640 |
| No treatment vs. 50x PMB | -9.625 | -14.87 to -4.376 | Yes | *** | 0.0003 |
| No treatment vs. 50x Colistin | -8.6 | -13.85 to -3.351 | Yes | *** | 0.0009 |
| 50x PMB vs. 50x Colistin | 1.025 | -4.224 to 6.274 | No | ns | 0.9960 |

74

75 **SUPPLEMENTARY MATERIAL AND METHODS**

76 **1.1. Extended spectrum β-lactamase (ESBL) phenotypic detection**

77 *P. aeruginosa* Ps4, *P. aeruginosa* PAΔD, *K. pneumoniae* K2 or *E. coli* E20 at 0.5
78 Mc Farland were inoculated in a plate of Mueller Hinton agar. Then, disks with
79 either ceftazidime (CAZ, 30 µg), ceftriaxone (CRO, 30 µg), cefotaxime (CTX, 30
80 µg), aztreonam (ATM, 30 µg) or the ESBL inhibitor clavulanic acid (AMC, 30 µg)
81 were placed on the surface of the agar to perform a double disk diffusion test.
82 Plates were incubated overnight at 37°C. Expansion of the inhibition halo towards
83 the inhibitor-containing disk located in the plate center indicates growth of an
84 ESBL-expressing organism.

85 To perform the combined disc test, Mueller Hinton agar plates inoculated with *K.*
86 *pneumoniae* CUN K2 or *E. coli* CUN E20 were incubated overnight at 37°C with
87 disks of ceftazidime (CAZ, 30 µg), ceftazidime/ clavulanic acid (CAZ/CLA, 30/10
88 µg), cefotaxime (CTX, 30 µg), cefotaxime/ clavulanic acid (CTX/CLA, 30/10 µg),
89 cefepime (FEP, 30 µg), cefepime/ clavulanic acid (FEP/CLA, 30/10 µg). An
90 increase of the inhibition halo surrounding the CLA containing disk with respect
91 to the disk lacking this inhibitor indicates growth of an ESBL-expressing
92 organism.

93 **1.2. Synergy testing by checkerboard and Bioscreen**

94 Briefly, a fresh culture of *P. aeruginosa* PAΔD, Ps4, Ps74 *E. coli* E20, *K.*
95 *pneumoniae* K2 was adjusted to 0.5 McFarland standard (equivalent to $10^8$
96 CFU/mL) and diluted 1:100 with MHCA to obtain a $10^6$ CFU/mL suspension.
97 Aliquots of 100 µL of this suspension were transferred into the wells of a standard
98 microtiter plate and mixed with an equal volume of antimicrobial solution. For

99 each strain, the antibiotic concentration range was selected according to
100 previously determined MICs. In total, 10 different concentrations of the selected
101 antibiotic (Amoxicillin, Ampicillin, Ticarcillin, Piperacillin or Ceftazidime) were
102 combined with 5 different concentrations of the BLI (oxacillin, phenylboronic acid,
103 aztreonam, tazobactam, clavulanic acid or sulbactam) maintaining the peptide
104 concentration (PMBN, P4-1, P4-5, P5-3, P5-5, P5-8, P5-9, P5-11, P5-12, P5-17,
105 P5-19). Microplates were incubated at 37°C and growth in the wells was visually
106 assessed after 18–20 h. Each assay included growth control wells containing
107 inoculated medium without antimicrobials and sterility control wells consisting of
108 uninoculated medium. The fractional inhibitory concentration index (FICI) for
109 each double (Equation (1)) or triple (Equation (2)) antimicrobial combination was
110 calculated as follows.

111 Equation (1) $FICI_{A/B} = \frac{MIC_{A\,(combination)}}{MIC_{A\,(alone)}} + \frac{MIC_{B\,(combination)}}{MIC_{B\,(alone)}}$

112 Equation (2) $FICI_{A/B/C} = \frac{MIC_{A\,(combination)}}{MIC_{A\,(alone)}} + \frac{MIC_{B\,(combination)}}{MIC_{B\,(alone)}} + \frac{MIC_{C\,(combination)}}{MIC_{C\,(alone)}}$

113 FICIs were calculated with the concentrations in the first non-turbid well found in
114 each row and column of the microplate. Combinations were classified as
115 synergistic (FICI ≤0.5), indifferent (0.5 < FICI ≤4), and antagonistic (FICI > 4).

116 Bioscreen C monitors the turbidity of bacterial cultures growing in 100-well
117 honeycomb plates at regular intervals. A cell suspension from an overnight
118 culture of *P. aeruginosa* Ps4, *E. coli* E20 or *K. pneumoniae* K2 was first adjusted
119 to $10^8$ CFU/mL and then diluted 100 times in the same broth and mixed with the
120 different treatments under study (two and three-component combinations, as well

121 as each antimicrobial alone). Microplate wells were filled with 200 µL of the test
122 suspensions and incubation was carried out at 37 °C for 48 hours with continuous
123 shaking and monitoring the absorbance every 15 minutes at 420-580 nm.

124 **1.3. Biofilm formation and assessment of anti-biofilm activity**

125 Briefly, a dense biofilm (1 x $10^{12}$ CFU/$cm^2$, approximately) was developed on the
126 surface of small disks called coupons that were constantly bathed in fresh TSB
127 medium. An overnight culture was inoculated into the reactor and incubated
128 without flow for 24h to ensure an initial adhesion of the bacteria. After additional
129 24 h of incubation under continuous flow, coupons were removed from the
130 chamber and planktonic cells were eliminated by rinsing them with phosphate
131 buffer (625 µM $KH_2PO_4$, 2 mM $MgCl_2 \bullet 6H_2O$, pH 7.2). Then, the coupons with 48
132 h mature biofilms attached to their surface were immersed in and treated with
133 1.75 mL of phosphate buffer, containing either a positive control of bactericidal
134 activity (chlorine 1,000 µg/mL), a standard treatment for Gram-negative
135 biofilms[37] (ceftazidime 5,000 µg/mL) or different combinations of the
136 antimicrobials at 50 times their planktonic MIC. Finally, coupons were incubated
137 at 37 °C for 72 h with renewal of the solution every 24 h and then they were rinsed
138 with phosphate buffer and processed for colony counting.

139 For the colony counting method, biofilms were detached by scraping the coupon
140 surface with a sterile wooden stick. Then, biofilm cells were suspended in
141 phosphate buffer, samples were homogenized by sonication for 5 min (Fungilab
142 US1'6; Spain) and aliquots were plated for counting. These count values were
143 used to calculate the so called, log density of the coupon which corresponds to
144 the CFU/$cm^2$ of biofilm cells attached to the coupon. In turn, log density allowed

145 the determination of $log_{10}$ reduction, which was defined as the difference of log
146 density between the untreated and the treated biofilm.

147 For the microscopic assessment of biofilm formation, biofilms grown on coupons
148 were first stained with the LIVE/DEAD BacLight kit (Life Technologies) following
149 the manufacturer's recommendations. Then, the coupon surface was examined
150 with a confocal laser microscopy (Leica TCS-SP5) using the FITC and TRITC
151 filters, a 1.5x objective and Imaris® software (Bitplane, Switzerland) [30].

152 **1.4. Killing curve and NPN uptake assays**

153 100 µL of an overnight culture of *P. aeruginosa* Ps4 were inoculated in 10 mL of
154 MHCA and grown at 37°C and 150 rpm until mid-log phase was achieved (3
155 hours). Then, four tubes with 10 mL of MHCA previously tempered to 37 °C were
156 inoculated with 50 µL of the inoculum adjusted to $5 \times 10^7$ CFU/mL. Antimicrobial
157 combinations were added at their MIC, 2xMIC and 4xMIC. A tube without
158 antimicrobials was also added as a control. Samples were collected at different
159 time points (0, 15, 30, 45, 60, 120, 240 and 360 min) and plated in TSA for colony
160 counting after serial dilution. Agar plates were incubated overnight at 37 °C and
161 $log_{10}$ CFU/mL were calculated.

162 A fresh culture of Ps4 in LB in logarithmic phase ($OD_{600nm}$=0.5) was centrifuged
163 for 10 min at 1000 g and at 26ºC. The sediment was resuspended in 5 mL of 5
164 mmol/L HEPES (Sigma-Aldrich) pH 7.2 at 37ºC. The suspension was stabilized
165 for 10 min at 37ºC and 100 µL were added to the wells of a dark microtiter plate
166 (Thermo Scientific). 100 µL of the antimicrobial previously diluted in HEPES at
167 37ºC was then added to the wells. After adding NPN (final concentration of 10

168 μM), the fluorescence was measured in a BMG Labtechnologies FLUOstar
169 Galaxy fluorimeter every 90 s and with an excitation and emission wavelength of
170 340 and 410 nm respectively with a bandwidth of 2.5 nm.

171 Wells with and without NPN in each one of the separate components were used
172 as controls.

173 **Supplementary Table 1.- Solvents used for the compounds under study.**

| Antimicrobial agent | Solvent |
|---|---|
| Amoxicillin | Phosphate buffer pH 6.0, 0.1M |
| Ampicillin sodium salt | Water |
| Aztreonam | Saturated solution of sodium bicarbonate |
| Ceftazidime hydrate | Water |
| Clavulanic acid | Water |
| Colistin | Water |
| Oxacillin | Water |
| Phenylboronic acid | 25%DMSO |
| Piperacillin sodium salt | Water |
| PMBN | Water |
| PMB | Water |
| Sulbactam | Water |
| Tazobactam | Water |
| Ticarcillin disodium salt | Water |
| Lactoferricin-derived peptides | Water |
| (P4-1, P4-5, P5-3, P5-5, P5-8, P5-9, P5-11, P5-12, P5-17 and P5-19) | |

174

175 Supplementary Table 2.- PMBN does not sensitize the colistin resistant strain

176 *Pseudomonas aeruginosa* Ps74 to ceftazidime and β-lactamase inhibitors.

| PMBN (mg/L) | MIC[1] of ceftazidime (mg/L) in the presence the mentioned concentration of inhibitor | | | | |
|---|---|---|---|---|---|
| | 0 | Aztreonam (1 mg/L) | FICI[2] | Phenylboronic acid (2 mg/L) | FICI |
| 0 | 8 | 8 | | 4 | |
| 1 | 8 | 8 | 1.03 | 4 | **0.50** |
| 2 | 16 | 8 | 1.04 | 4 | 0.51 |
| 4 | 16 | 8 | 1.04 | 4 | 0.51 |
| 8 | 16 | 16 | 2.05 | 8 | 1.02 |
| 16 | 16 | 16 | 2.06 | 4 | 0.53 |

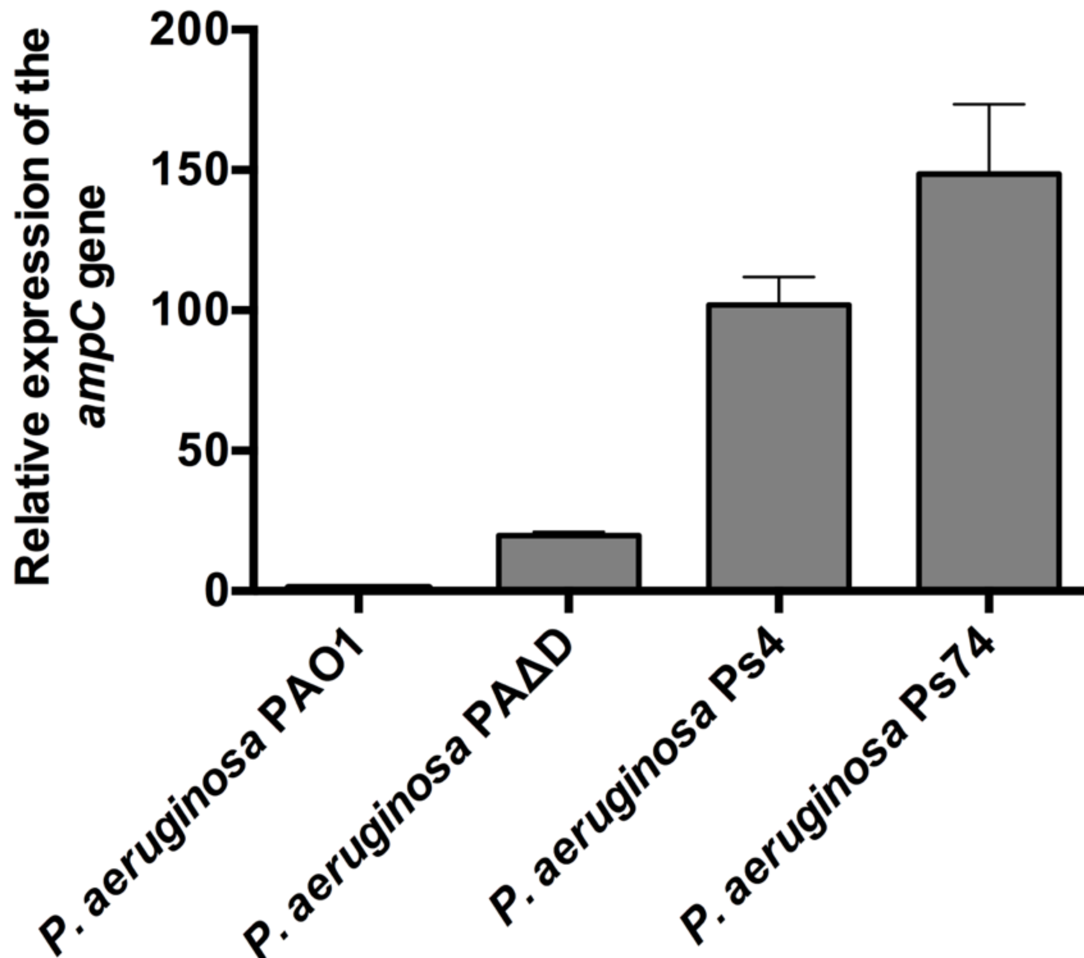
177 [1]: Minimum inhibitory Concentration. [2]: Fractional inhibitory concentration index.

178 Synergistic combinations (FICI<0.5) are indicated in bold. MIC $_{Ceftazidime}$= 8 mg/L.

179 MIC $_{Aztreonam}$= 32 mg/L. MIC $_{Phenylboronic\ acid}$ >512 mg/L. MIC $_{PMBN}$= 512 mg/L. MIC

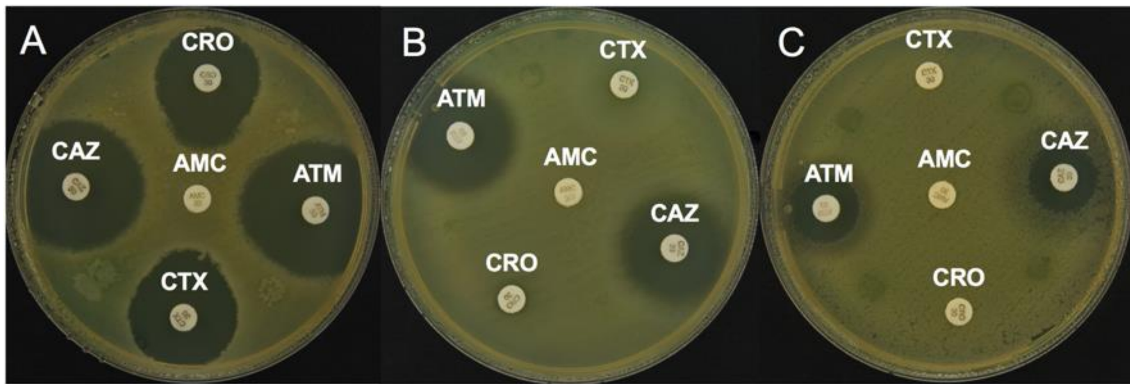
180 $_{Colistin}$= 32 mg/L.

181



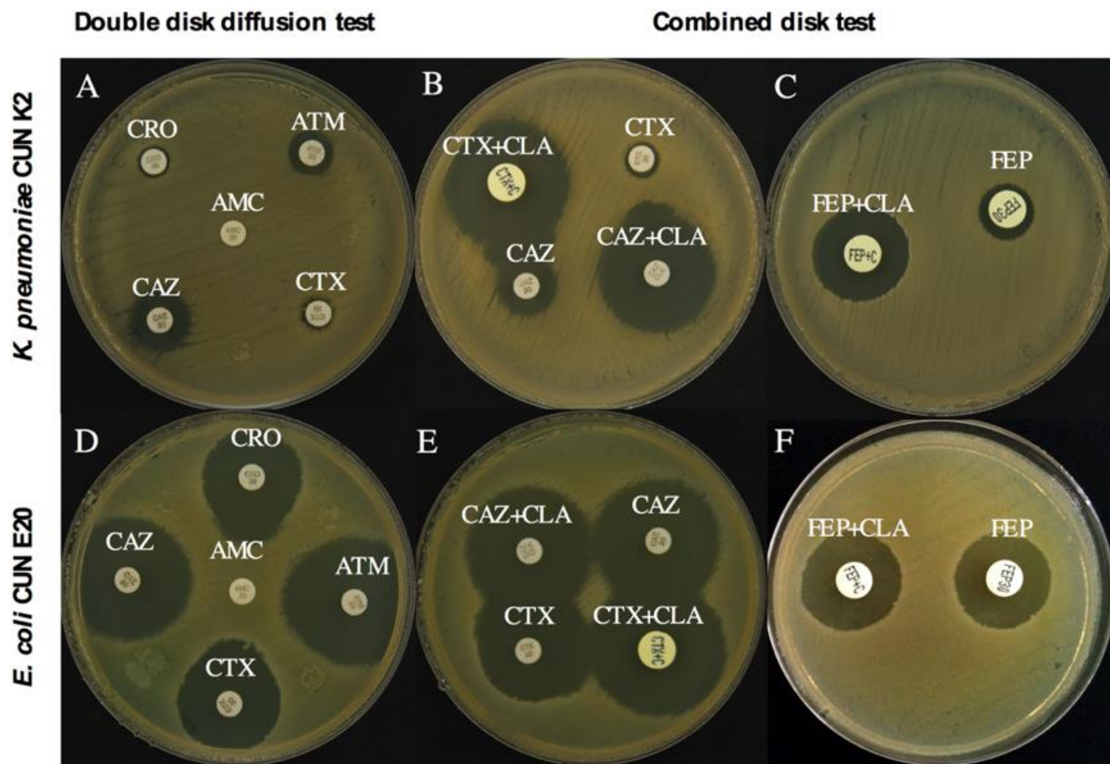

182

183 **Supplementary Figure 1.- *Pseudomonas aeruginosa* PAΔD, Ps4 and Ps74**
184 **overexpress the gene *ampC*** as determined by RT-qPCR. Results shown are the
185 means ± standard error of three independent experiments where each strain was
186 tested in triplicate (n=9). Levels of *ampC* are expressed relative to those measured
187 in *P. aeruginosa* PAO1 (reference value= 1).

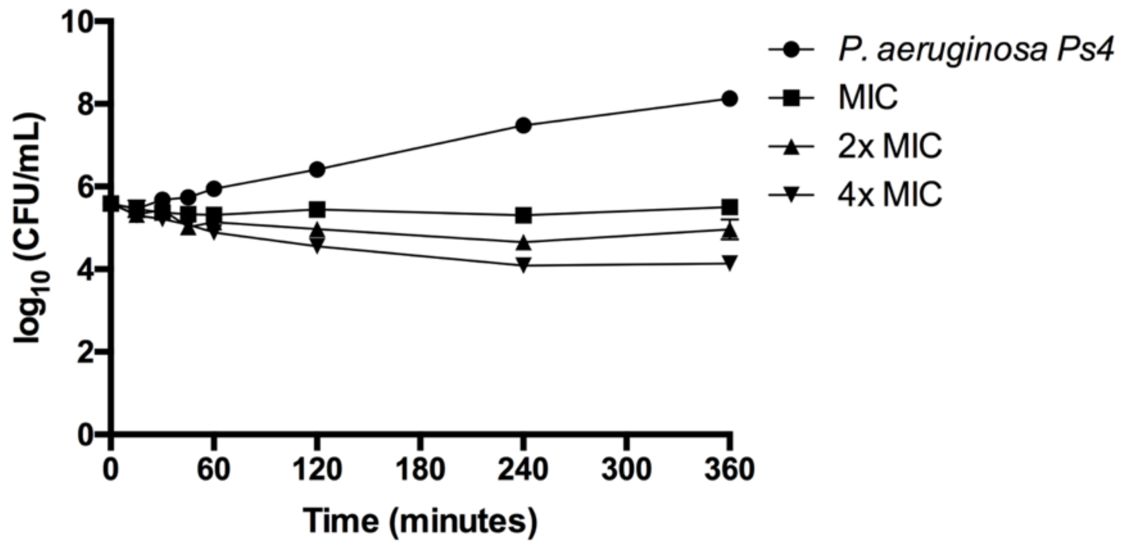
188 


189 **Supplementary Figure 2.- Double disk diffusion test for the strains A)**
190 ***Escherichia coli* CUN E20 B) *Pseudomonas aeruginosa* PAΔD and C)**
191 ***Pseudomonas aeruginosa* Ps4**. Image A shows that the inhibition area caused
192 by the antibiotics ceftazidime (CAZ), aztreonam (ATM), ceftriaxone (CRO) and
193 cefotaxime (CTX) expands towards the disk containing amoxicillin/clavulanic acid
194 (AMC), an Extended spectrum β-lactamase inhibitor. This indicates that CUN E20
195 expresses ESBL. Strains PAΔD and Ps4 do not exhibit this behavior.

196




197 **Supplementary Figure 3.- Growth of the clinical strains *Klebsiella*
198 *pneumoniae* CUN K2 and *Escherichia coli* CUN E20 in Double disk diffusion
199 test plates (A and D) and combined disk test (B, C, E and F).** (B) and (C)
200 *Klebsiella pneumoniae* CUN K2 sensitivity to cephalosporins increases when the
201 antibiotic-containing-disk is supplemented with clavulanic acid. This was also
202 detected in *E. coli* (E) and (F) to a lesser extent because of its high sensitivity to
203 the uncombined antibiotic. (D) The clavulanic acid-containing central disk
204 enhances *Escherichia coli* CUN E20 sensitivity to β-lactams causing the inhibitory
205 halo to expand towards the center). This phenomenon passed unnoticed in
206 *Klebsiella pneumoniae* (A), due its low antibiotic susceptibility. Antibiotics are
207 ceftazidime (CAZ), aztreonam (ATM), ceftriaxone (CRO), cefepime (FEP),
208 cefotaxime (CTX) and amoxicillin/clavulanic acid (AMC), some disks are
209 combined with clavulanic acid (CLA).




**Supplementary Figure 4.- Killing curve of *Pseudomonas aeruginosa* Ps4 grown with different concentrations of the same antimicrobial combination.** The MIC corresponded to ceftazidime (0.06 mg/L), phenylboronic acid (2 mg/L) and PMBN (4 mg/L). Samples were taken at 0, 15, 30, 45, 60, 120, 240 and 360 min. Results shown are the means ± standard error of three independent experiments where each condition was tested in duplicate (n=6).