Future MICROBIOLOGY

Short Communication



# Bactericidal synergism between antibiotics and phage endolysin Cpl-711 to kill multidrug-resistant pneumococcus

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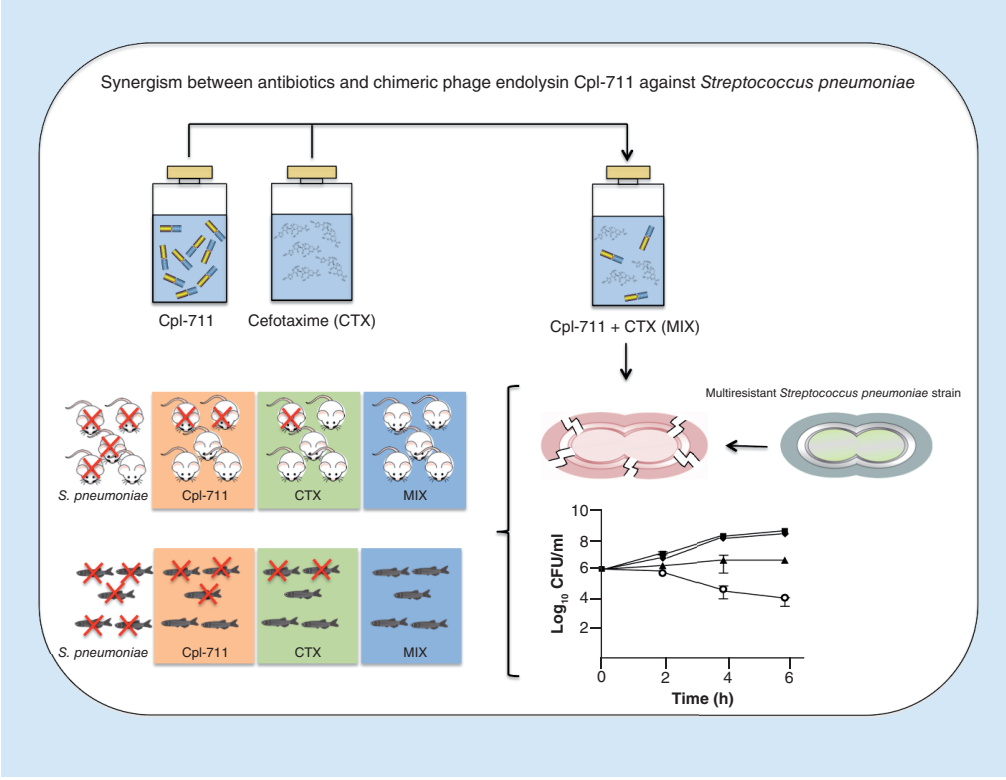
**Aim:** To test the synergistic effect of Cpl-711 endolysin and antibiotics for antipneumococcal activity. **Materials & methods:** A combination of Cpl-711 and different antibiotics (amoxicillin, cefotaxime, levofloxacin and vancomycin) was tested in a checkerboard assay against several multidrug-resistant *Streptococcus pneumoniae* strains. Mouse and zebrafish models of pneumococcal sepsis were used to confirm the *in vitro* data. **Results:** The activity of Cpl-711 combined with amoxicillin or cefotaxime was synergistic in the bactericidal effect against a serotype 23F multiresistant clinical isolate of *S. pneumoniae*. Synergy between Cpl-711 and cefotaxime was validated using both mouse and zebrafish models. **Conclusion:** Combination of Cpl-711 and cefotaxime may help in the treatment of diseases caused by multiresistant pneumococcal strains.

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**Keywords:** antibiotic multidrug-resistant • endolysin • mouse model • *Streptococcus pneumoniae* • synergy • zebrafish model

*Streptococcus pneumoniae* is a common etiological agent of serious respiratory infections and invasive disease in children and adults, like otitis media, pneumonia, bacteremia and meningitis [1]. Thus, this pathogen constitutes a major cause of mortality worldwide that provokes a serious human health concern. This situation has worsened by the increase of multidrug-resistant pneumococcal isolates which are posing a real challenge for the treatment of these infections [2]. For these reasons, novel and improved therapeutics against *S. pneumoniae* and other multidrug-resistant pathogens are needed. One promising approach is based on bacteriophage endolysins and peptidoglycan hydrolases synthesized as part of the tightly controlled phage late genes, which normally help mature phage particles to lyse the host bacteria. These enzymes are very efficient at breaking specific bonds of bacterial peptidoglycan from the inside of the host cell, but its rapid action can also be achieved when added exogenously as purified proteins. Such enzymes, also called enzybiotics, have attracted considerable interest as novel antimicrobials, mostly against Gram-positive bacteria, and have been used to fight bacterial infections and prevent pathogen colonization of mucous membranes in animal models [3]. Cpl-711 is a chimeric enzyme constructed by the fusion of two pneumococcal phage lysozymes belonging to the GH25 family of glycosyl-hydrolases, in other words, the catalytic domain comes from Cpl-7 and the cell wall binding domain comes from Cpl-1 [4,5]. As a result, Cpl-711 is a choline binding protein and has shown to be the most active and specific endolysin against pneumococci described to date, capable of protecting mice challenged with the D39_IU pneumococcal strain by treatment with a single intraperitoneal (ip) injection of Cpl-711 [5]. The synergistic effect of a combination of different endolysins has been previously described [6,7], as well as the mixture of antibiotics and lytic enzymes [8–10]. This study aimed to determine the *in vitro* effect of the simultaneous use of Cpl-711 and amoxicillin (AMX), cefotaxime (CTX), levofloxacin (LFX), or vancomycin (VAN) against four different multiresistant strains of *S. pneumoniae*, together with validation of the results in animal models.

## Materials & methods

### Bacterial strains, antibiotics & endolysin

The pneumococcal strains used in this work and their MICs are shown in Table 1. The Cpl-711 endolysin was purified from the *Escherichia coli* BL21 (DE3) [pTRD762] strain, as previously described [5].

### Checkerboard & isobologram analysis

Checkerboard tests were assessed in triplicate by the microdilution method, as previously described [11,12]. All compounds were tested at six concentrations, at two-fold serial dilutions, which usually ranged from 0.06 × to 2 × MIC. Each microtiter well contained 100 µl of a pneumococcal inoculum of $1 \times 10^5$ colony forming units (CFUs)/ml, with or without the corresponding antibiotics, in a final volume of 200 µl of cation-adjusted Mueller Hinton broth supplemented with 5% lysed horse blood per well and the plates were incubated at 37°C for 22 h.

The fractional inhibitory concentration index (FICI) was calculated as the MIC of Cpl-711 or each antibiotic in the combination, divided by the MIC of the Cpl-711 or each antibiotic alone [11,12]. The FICI was obtained by the sum of FICIs, as follows:

$FICI_X$ $FICI_A + FICI_B$ $MIC_A$ in combination/$MIC_A$ + $MIC_B$ in combination/$MIC_B$

The MIC of drug A is marked on the *x*-axis of an isobologram and the MIC of drug B on the *y*-axis. The line connecting these two data is the indifference line (no interaction). The different FICI values of the combination indicate additive ($1 \geq FICI > 0.5$), synergistic ($FICI \leq 0.5$), indifferent ($1 < FICI \leq 4$) or antagonistic ($FICI > 4$) interactions [12].

### *In vitro* time-killing curve tests of single drugs & combinations

Time-killing assays were assessed according to the Clinical & Laboratory Standards Institute (CLSI) guidelines [13]. The studied combinations in time-killing assays were carried out with each antibiotic and enzyme alone or in combination, at proven concentrations of synergistic effect. In these assays, $1 \times 10^6$ CFU/ml of the tested strains were incubated in 3 ml of cation-adjusted Mueller Hinton broth supplemented with 5% lysed horse blood with the individual compounds or the combinations in separate tubes. At intervals of 2, 4 and 6 h, aliquots were removed

Table 1. MICs and fractional inhibitory concentration indexes for combination treatments with Cpl-711.

| | | Strain name[†] | | | |
|---|---|---|---|---|---|
| | Antibiotic | D39 (2) | 48 (23F) | 450 (11A) | 3498 (8) |
| **MIC (mg/l)** | Amoxicillin | 0.03 | 16 | 8 | 0.03 |
| | Cefotaxime | 0.03 | 16 | 1 | 0.03 |
| | Levofloxacin | 0.50 | 2 | 0.50 | 32 |
| | Vancomycin | 0.40 | 0.40 | 0.40 | 0.20 |
| | Cpl-711 | 1 | 1 | 1 | 2 |
| **FICI for Cpl-711 and:**[‡] | Amoxicillin | 0.43[§] | 0.50[§] | 0.31[§] | 0.91[¶] |
| | Cefotaxime | 0.50[§] | 0.50[§] | 0.62[¶] | 0.87[¶] |
| | Levofloxacin | 0.40[§] | 0.50[§] | 0.62[¶] | 0.75[¶] |
| | Vancomycin | 0.31[§] | 0.50[§] | 0.75[¶] | 0.50[§] |

[†] Serotype is indicated in parentheses.
[‡] Values are based on a 0.25 × MIC for Cpl-711.
[§] Synergistic combinations.
[¶] Additive combinations.
FICI: Fractional inhibitory concentration index.

from each tube and diluted serially (1:10) using sterile saline to determine cell viability. Experiments finished at 6 h due to the autolysis induced by β-lactam antibiotics. From each dilution, 10 µl were added in tryptic soy agar plates containing 5% defibrinated sheep blood and were incubated at 37°C for 24 h (detection limit, $10^2$ CFU/ml). According to Clinical & Laboratory Standards Institute, a combination of two antimicrobial agents is considered synergistic when it causes a ≥2 log unit reduction in CFU/ml, compared with the sum of the reductions observed with the individual compounds at the end of the experiment, in this case at 6 h [13].

### Mouse model of *S. pneumoniae* bacteremia

Experimental procedures involving mice were performed at Instituto de Salud Carlos III conforming to the Spanish government legislation (RD 53/2013) and European Community regulations (2010/63/EU). BALB/c female mice (8–12 weeks old) weighing about 20 g were purchased from Harlan Laboratories Models (Barcelona, Spain). These mice have been shown to be a good animal model to explore the protective activity of several antibiotics and enzybiotics against sepsis [2,5,14]. Animal experiments were performed in groups of at least four mice and were repeated twice. The sepsis infection model was based on methods described previously [2,5], and carried out using 4–6 week-old female BALB/c mice. To determine the subtherapeutic doses, a preliminary experiment was performed using groups of two mice challenged with different doses of CTX and Cpl-711 to evaluate the protective activity of increasing concentrations (data not shown). Briefly, mice were infected by the ip. route with 0.2 ml of a $5 \times 10^8$ CFU/ml suspension of *S. pneumoniae* 48 strain. One hour post infection, treatment was initiated by the subcutaneous route with a subtherapeutic single dose of CTX (25 mg/kg) or Cpl-711 (4 µg/mouse) alone or in combination, or 20 mM sodium phosphate, 150 mM NaCl, pH 6.0 phosphate-buffered saline (PBS) buffer in the control group. Bacterial levels in blood, from the tail vein, were determined from every infected mouse (control and treated groups) at 24 h postinfection, when the majority of mice were alive, as previously described [5,15].

### Zebrafish model of *S. pneumoniae* infection

Zebrafish (n = 10 per condition) were infected by ip. route with 10 µl of $5.5 \times 10^5$ CFU/ml suspension of *S. pneumoniae* strain 48. One hour postinfection, adult zebrafish were divided into four treatment groups and were treated by the ip. route with subtherapeutic doses of CTX (25 mg/kg) or Cpl-711 (0.13 mg/kg) alone or in combination, or using PBS as control. The survival rate for each experimental group was monitored every 24 h for up to 4 days postinfection. In addition, to investigate the protective effect, we further examined the numbers of bacterial CFUs in blood in a preclinical sepsis model after 24 h posttreatment. One day postchallenge, blood was collected from the caudal fin, 2 µl, and the number of bacteria was determined.

### Statistical analysis

All *in vitro* results are representative of data obtained from repeated independent experiments, and each value represents the mean ± standard deviation for four replicates. Statistical analysis was performed by using the two-tailed Student's t-test (for two groups), whereas analysis of variance was chosen for multiple comparisons. For all *in*

*vivo* data, the log-rank (Mantel–Cox) and Gehan–Breslow–Wilcoxon tests were used to draw, analyze and compare the survival curves. GraphPad InStat version 5.0 (GraphPad Software, CA, USA) was used for statistical analysis.

## Results

### Synergistic effect of the endolysin & antibiotics

To test the bactericidal effect of Cpl-711 endolysin combined with antibiotics, some of the drugs most commonly used against invasive pneumococcal disease were chosen [16,17]. These antibiotics belong to different groups including two β-lactams (AMX and CTX), a fluoroquinolone (LFX) and a glycopeptide (VAN). As pneumococcal strains, we selected the fully susceptible D39 strain (serotype 2) and three resistant clinical isolates such as the 48 strain (serotype 23F; resistant to β-lactams, tetracycline and erythromycin), the 450 strain (serotype 11A; resistant to β-lactams) and the 3498 strain (serotype 8; resistant to LFX, tetracycline and erythromycin) (Table 1). Of note, the MIC of Cpl-711 (1–2 μg/ml) is rather constant for each of the strains tested, about one or two-log units lower than that of Cpl-1 (25–512 μg/ml) [8,9]. The results of checkerboard *in vitro* studies testing the combinations used (0.25 × MIC for Cpl-711 and antibiotics) are summarized in Table 1, and the isobolograms for each combination of endolysin and antibiotic are shown in Figure 1. These data demonstrated both synergistic and additive effects, depending on the particular antibiotic and strain tested. Interestingly, none of the combinations produced indifferent or antagonistic effects. Synergism was seen using AMX, CTX and LFX in combination with 0.25 × MIC of Cpl-711, as in strains D39 and 48. In the case of strains 450 and 3498, synergistic values were only found with AMX and VAN, respectively, in combination with 0.25 × MIC of Cpl-711 (Table 1 & Figure 1). It is noteworthy that all combinations tested between Cpl-711 and any antibiotic showed synergism against the susceptible strain D39 and the resistant isolate 48.

### Time-killing analysis of drug combinations against pneumococcal strains

To confirm the possible synergistic activities of Cpl-711 with AMX, CTX, LFX and VAN, time-killing assays were performed against the pneumococcal strains showing FICIs ≤0.5 for each drug combination. The range of antibiotics and Cpl-711 concentrations was determined according to the checkerboard and isobologram results. These concentrations were used for each treatment, either with a single agent or in combination. The results demonstrated that the combinations with the most effective synergistic activity against log-phase bacteria were those involving AMX and CTX, which are shown in Figure 2 (data with LFX and VAN are not shown). When combined 0.25 × MIC Cpl-711 with 0.25 × MIC AMX, the viable cells were reduced about 5-log units at 6 h posttreatment in the case of strain D39 and almost 2-log units for strain 450 (Figure 2A & C), whereas in the other strains this combination was ineffective in terms of synergistic effect. For the clinical isolate 48 (serotype 23F), only the combination of 0.06 × MIC of Cpl-711 and 0.5 × MIC of CTX presented a synergistic effect, since the viability was reduced about 4-log units at 6 h posttreatment (Figure 2F).

### *In vivo* combined activity of cefotaxime & Cpl-711 in a mouse infection model

To test whether the combination of one antibiotic and Cpl-711 could prevent death from pneumococcal infection, a mouse model of bacteremia by ip. infection with 48 strain was set up. Experiments were performed on different days, and mice survival analysis and bacterial counts in blood are shown in Figure 3A,B. Mice were infected with a lethal dose of strain 48 and 1 h later PBS was added to the control group whereas Cpl-711 (4 μg/mouse), CTX (25 mg/kg) or the combination of Cpl-711 and CTX were administered to groups of at least four mice. Survival was monitored during 7 days and bacterial counting from blood was obtained at 24 h. Mice were challenged with a high dose of strain 48, by the ip. route, but only caused the death of 47% of the animals. The low virulence in mice may be related to high level of resistance of this particular strain to β-lactam antibiotics (MIC 16 μg/ml), as this correlation has been demonstrated before [18]. Treatment with 25 mg/kg of CTX or 4 μg of Cpl-711 partially protected 67% of mice (6/9) or 58% of animals (11/19), respectively, compared with 53% of the control group (9/17). Nevertheless, 100% survival was achieved with a combination of both compounds at the same dosages (Figure 3A). In addition, the bacterial load in the blood of mice, at 24-h postchallenge, correlated with these protection numbers. Animals treated with CTX or Cpl-711 alone had lower bacterial counts in blood compared with the control group, whereas mice treated with the combination of CTX and Cpl-711 showed significant reductions of bacterial levels in blood when compared with both control and individual treatments. Moreover, a significant increased proportion of mice cleared the bacteria completely in the combination group in comparison with the individual group ($p < 0.01$) (Figure 3B).

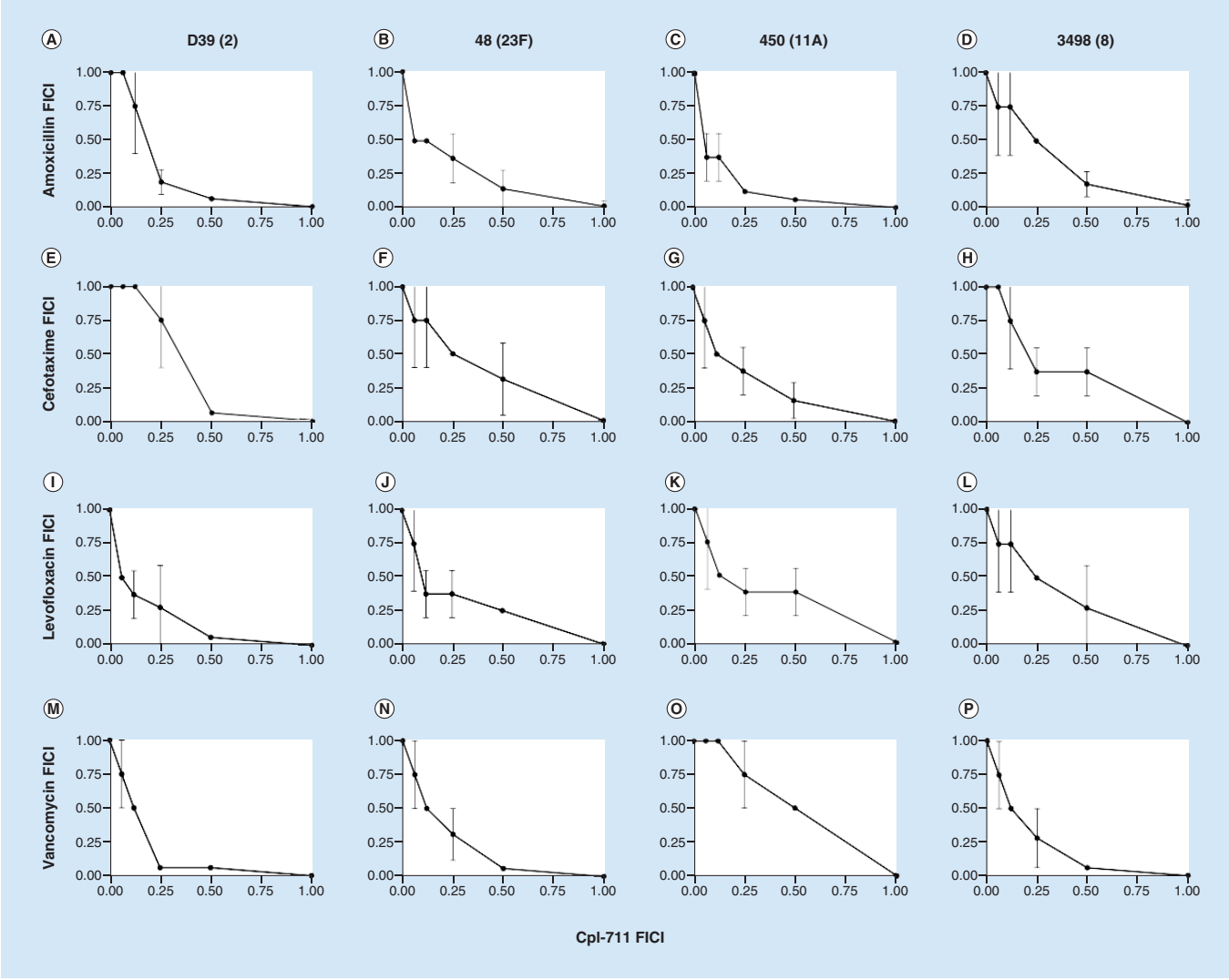


**Figure 1. Isobolograms of the checkerboard synergy testing methods. (A–D)** Four multiresistant strains were tested with combinations of Cpl-711 and amoxicillin. Strains D39, 48 and 450 were killed synergistically; strain 3498 was not quite killed synergistically. **(E–H)** The same strains were tested with combinations of Cpl-711 and cefotaxime. Only D39 and 48 were killed synergistically. **(I–L)** The same strains were tested with combinations of Cpl-711 and levofloxacin. Only D39 and 48 were killed synergistically. **(M–P)** The same strains were tested with combinations of Cpl-711 and vancomycin. D39, 48 and 3498 were killed synergistically. Error bars show standard deviations.

### *In vivo* combined activity of cefotaxime & Cpl-711 in a zebrafish infection model

Zebrafish is becoming a frequent model to test infections, ranging from bacterial or viral to fungal pathogens. As these animals present adaptive immune response at 4–6 weeks, after this period, it is possible to carry out studies to analyze antimicrobial agents [19]. Thus, we set up the protocol and divided the zebrafish into the same type of groups as explained in the previous mouse model. Then, animal survival was determined at different times. All zebrafish deaths due to the strain 48 infection occurred during the first day. The treatment with CTX and Cpl-711 alone showed only partial levels of protection, from 45 (18/40) to 23% (9/40), respectively. Nevertheless, the combination of both drugs fully protected zebrafish with a 100% survival (Figure 3C). Concerning the bacterial counting, it was not possible to calculate the value in the infected, but untreated, zebrafish due to their rapid death. Zebrafish treated only with CTX or Cpl-711 gave an average of $2.4 \times 10^4$ and $2.5 \times 10^4$ CFU/ml 24 h postchallenge, respectively, whereas the average of the bacterial titer of zebrafish treated with the combination of both compounds significantly diminished to 20 CFU/ml ($p < 0.01$) (Figure 3D). These results confirmed the validity of synergistic effect between an endolysin, Cpl-711 and an antibiotic, CTX, carried out in mouse and zebrafish models of infection.

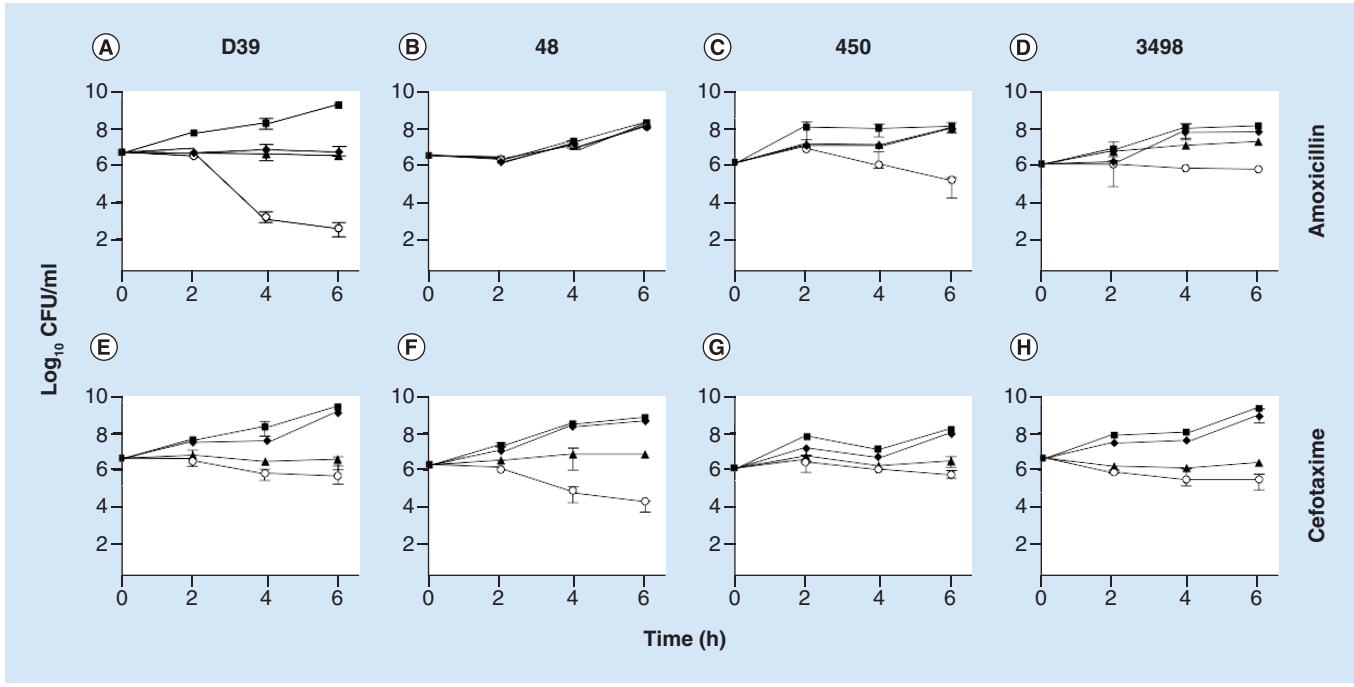


**Figure 2. Time-killing assays with pneumococcal strains. (A–D)** Four different *Streptococcus pneumoniae* strains were treated with phosphate-buffered saline as control (squares) or with 0.25 × MIC of Cpl-711 (filled diamonds), 0.25 × MIC of AMX (filled triangles) or a combination of 0.25 × MIC of each (open circles) for 6 h. **(E–H)** The same strains were treated with PBS as control (squares) or with 0.06 × MIC of Cpl-711 (filled diamonds), 0.5 × MIC of CTX (filled triangles) or a combination of 0.06 × MIC of Cpl-711 and 0.5 × MIC of CTX (open circles) for 6 h.

## Discussion

Exploring combinations of two or more antibacterials can be useful because they may act synergistically and, thus, it is an effective way to improve the bactericidal activity of individual drugs. In this work, we have combined different concentrations of the potent and specific endolysin Cpl-711 with relevant antibiotics used clinically against pneumococcal infections to look for a possible synergy. Initial checkerboard experiments suggested a synergistic effect for most of the combinations and strains used; although this effect was not always confirmed when time-killing assays were carried out. This variability between both types of experiments has been already reported [10] and it is not fully understood, but a possible explanation in this particular case could be the different initial bacterial inoculum between the two tests (around ten-times more cells in a time-killing experiment than in a checkerboard protocol). In addition, checkerboard measurements are usually made at one time point and therefore do not give a dynamic view of the antimicrobial interactions.

Overall, time-killing assays of four antibiotics against one susceptible and three multiresistant strains showed clear synergistic effects when AMX and CTX were combined with Cpl-711 against strains D39, 48 and 450. Nevertheless, the reasons explaining why such effect is produced only at some concentrations and appears to be restricted to particular strains are not known. In this sense, other authors only observed synergism between a β-lactam antibiotic (penicillin) and the endolysin Cpl-1, against a highly resistant pneumococcal strain [8], suggesting that enzybiotic/penicillin synergism could increase somehow with β-lactam resistance. This does not seem to be the case in our study since synergy appears to be independent of antibiotic susceptibility. As for the mechanism by which synergy occurs, in the particular case between two endolysins with different catalytic specificity, the observed synergy is normally explained by an enhanced destructive effect when the enzymes cleave two different bonds at the same time within the three-dimensional peptidoglycan meshwork. Nevertheless, the mechanism of synergistic effect between endolysins and antibiotics remains largely unclear. It is possible that endolysins may enhance antibiotic activity in a similar way as peptidoglycan cleavage could facilitate antibiotic uptake, but it is also plausible that an altered peptidoglycan induced by antibiotic treatment would be more susceptible for endolysin action. In fact, it has been speculated that synergy between an endolysin and β-lactam antibiotics could be related with their mechanism of action [8]. Concerning *S. pneumoniae*, it has been well established that alterations in the

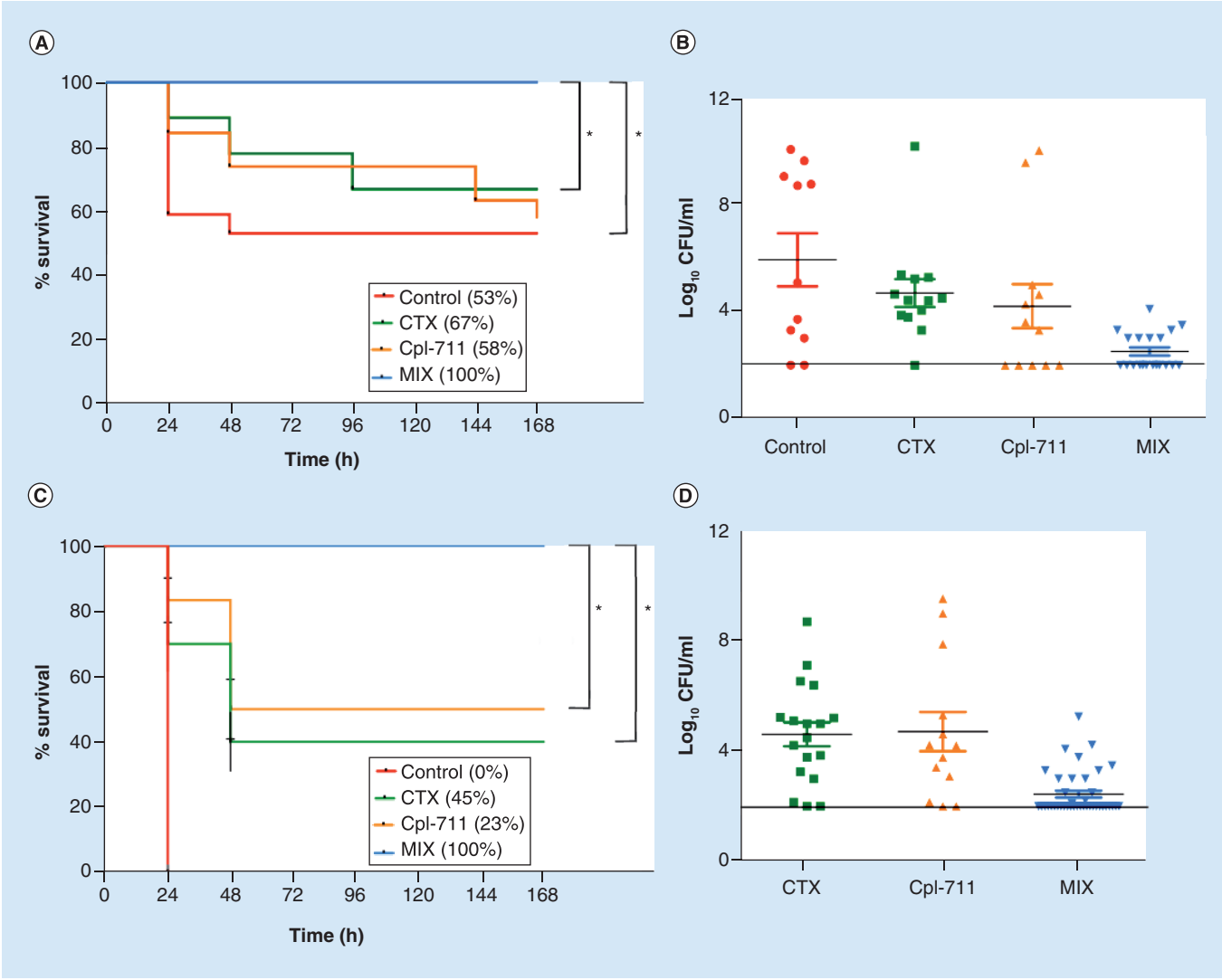


**Figure 3. Protection by administration of CTX, Cpl-711 and the combination (MIX) against pneumococcal sepsis in mice and zebrafish.** **(A)** Survival of mice infected with strain 48 and treated with PBS as control or with 25 mg/kg of CTX, 4 μg of Cpl-711 or a combination of both, monitored for a period of 7 days (168 h). **(B)** Mice bacterial counts obtained at 24 h postchallenge, where each figure represents the bacterial titer of an individual. **(C)** Survival of zebrafish infected with strain 48 and treated with PBS as control or CTX (25 mg/kg), 0.13 mg/kg of Cpl-711 or a combination of both, monitored for a period of 7 days. **(D)** Zebrafish bacterial counts obtained at 24 h postchallenge, where each figure represents the bacterial titer of an individual. In panels B and D, horizontal bars indicate the median bacteria titer. Statistical analyses were performed using a t-test for two comparisons and ANOVA test (*$p < 0.01$).

target enzymes for β-lactam antibiotics and penicillin-binding proteins, constitute a major resistance mechanism in this pathogen [20]. The inhibition of peptidoglycan synthesis precludes bacterial elongation and division, which results in bacterial cell lysis due to osmotic stress [21]. In this scenario, the endolysin, which directly attacks and compromises the cell wall structure, could both increase antibiotic accessibility to its binding site and collaborate with β-lactams in cell wall erosion, although this mechanism remains to be experimentally proved.

An accepted approach to treat infections produced by multiresistant bacteria, is using a combination of antimicrobial drugs, especially those showing synergistic effects, which could both hamper the emergence of resistance [22], and reduce its therapeutic relevance by increasing susceptibility [10]. In this regard, our data show that applying subinhibitory Cpl-711 concentrations against AMX- or CTX- resistant pneumococcal strains effectively diminishes the antibiotic concentration needed to reach an inhibitory (Table 1) or therapeutic effect (Figure 3).

Confirmation of *in vitro* results in an animal model of infection is important to assess the validity of the therapeutic efficacy, since it is not always that the synergism found in the combination of two compounds by

checkerboard and/or time-kill analysis is confirmed *in vivo*, as it was the case with two different endolysins (λSA2 and B30) with bactericidal activity against several streptococcal species [7]. On the other hand, positive *in vivo* synergistic effects have been reported combining Cpl-1 endolysin with gentamycin or penicillin [8], and also with Pal, another pneumococcal endolysin [6]. Besides, a strong *in vivo* evidence for the benefit of the combination of AMX with CTX in treatment of *Enterococcus faecalis* endocarditis has also been published [23]. Synergy *in vitro* data obtained in this work have been confirmed in two different animal models of infection, which reinforces the prospect of using specific combinations of Cpl-711 and CTX to resensitize pneumococcal strains to β-lactams, although it should need further validation in other strains. These cocktails should avoid the appearance of new resistances and significantly reduce the required dose and duration of the treatments.

## Conclusion & future perspective

Currently, phage endolysins cannot replace antibiotics as standard antimicrobials, but they may become valuable complement of antibiotics, especially to fight multiresistant pathogens. Due to this promising perspective, the number and variety of novel endolysins showing synergy with classic antibiotics is increasing and may translate into resensitization of bacteria to current antibiotics and into much lower doses of the individual agents being required for treatments. The synergistic activity of Cpl-711 endolysin with CTX, confirmed in two different animal models of infection, provides good hope that the combination of both drugs may help in fighting pneumococcal diseases.

### Summary points

- In this study, Cpl-711 endolysin, a chimeric murein hydrolase with the most killing activity against pneumococci, was explored for its potential improved action by combining with different antibiotics.
- Synergism was clearly demonstrated with a combination of Cpl-711 and amoxicillin or cefotaxime against several multiresistant strains.
- Synergistic bactericidal effect of Cpl-711 and cefotaxime was validated using mouse and zebrafish models infected with the pneumococcal multiresistant strain 48.
- The combined treatment of an endolysin and an antibiotic may be promising to combat infectious diseases provoked by multiresistant pneumococcal strains.

### Financial & competing interests disclosure

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No writing assistance was utilized in the production of this manuscript.

### Ethical conduct of research

Mice experiments were carried out in ISCIII animal facilities under strict accordance with the recommendations of the Animal Care and Use Committee of ISCIII (approval reference number CBBA-PA 52_2011-v2 and PROEX 218/15). Animal experiments conducted at Ikan Biotech in The Zebrafish Lab department were performed according to European Union guidelines for handling of laboratory animals (http://ec.europa.eu/environment/chemicals/lab_animals/home_en.htm). Signs of infection were monitored three times daily throughout the experimental time course. Moribund zebrafish were euthanized by immersion in unbuffered MS222 solution (250 mg/l; 25–30°C). Approval for these studies was granted by the University of Navarra's Ethics Committee for Animal Experimentation (Protocol 034-17). Experiments were carried out at The Zebrafish Lab's animal housing facility with all efforts made to minimize animal suffering.

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