Title

Efficacy and pharmacokinetics of the combination of OP0595 and cefepime in a mouse model of pneumonia caused by ESBL-producing *Klebsiella pneumoniae*

Running Title

Efficacy and pharmacokinetics of OP0595 in pneumonia

Authors

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Abstract

Background: OP0595 (RG6080) is a novel diazobicyclooctane that inhibits class A and C serine beta-lactamases. Although the combination of OP0595 and cefepime (FEP) showed good in vitro activity against extended spectrum beta-lactamase (ESBL)-producing pathogens, the effect of the combination therapy against severe infections, such as pneumonia or bacteraemia, remains unknown in vivo.

Objectives: In this study, we investigated the efficacy and pharmacokinetics of the combination therapy of OP0595 and FEP in a mouse model of pneumonia caused by *Klebsiella pneumoniae* harbouring SHV and CTX-M-9-type ESBLs.

Methods: The infected BALB/c mice were intraperitoneally administered saline (control), 100 mg/kg of FEP, 20 mg/kg of OP0595, or both FEP and OP0595, twice a day.

Results: The minimum inhibitory concentration (MIC) of FEP against the bacteria was 8 mg/L and markedly improved to 0.06 mg/L with the addition of 0.5 mg/mL of OP0595. In the survival study, the combination of FEP and OP0595 significantly improved the survival rate compared to that reported with either OP0595 or FEP alone ($P < 0.001$). The number of bacteria in the lungs and blood significantly decreased in the combination therapy group compared to that reported for the monotherapy groups ($P < 0.001$). In addition, the in vivo effect depended on the dose of FEP. However, pharmacokinetic analysis revealed that the percentage of time above MIC remained constant when increasing the dose of FEP in combination with 20 mg/kg of OP0595.

Conclusions: The results of our study demonstrated the in vivo effectiveness of the combination of OP0595 and FEP.

Key words: ESBLs; *Klebsiella pneumoniae*; serine-beta-lactamase inhibitor; diazobicyclooctane

Introduction

Extended-spectrum beta-lactamases (ESBLs) are enzymes classified as Class A in the Ambler classification of beta-lactams,(1) and are responsible for multi-resistance to most beta-lactam antibiotics including penicillins, cephalosporins, and monobactams. The global increase in ESBL-producing pathogens, particularly *Escherichia coli* and *Klebsiella pneumoniae*, is a major clinical concern. The current effective therapeutic option for severe infections caused by ESBL-producing pathogens is carbapenems.(2, 3) However, it is necessary to develop other therapeutic options because of the emergence and global spread of carbapenemase-producing Enterobacteriaceae.

Recently, combination agents consisting of a cephalosporin and beta-lactamase inhibitor, such as ceftazidime-avibactam and ceftolozane-tazobactam, have been developed. Ceftolozane-tazobactam showed good activity against most ESBL-producing *E. coli* isolates, but lower activity against ESBL-producing *K. pneumoniae* isolates.(4, 5) By contrast, the addition of avibactam significantly increased the activity of ceftazidime against ESBL-producing pathogens including *K. pneumoniae*.(6) OP0595 (RG6080) is a novel diazobicyclooctane that, similar to avibactam, inhibits class A and C serine beta-lactamases. In addition to the inhibition of beta-lactamases, OP0595 showed antimicrobial activity by inhibiting penicillin binding protein 2 (PBP2) and enhanced the antimicrobial activity of the beta-lactams that bind to PBP3.(7, 8) The combination of OP0595 and beta-lactams showed good in vitro activity against ESBL-, AmpC-, and carbapenemase-, including OXA-48 class beta-lactamases, producing pathogens.(7–9) Additionally, the combination of OP0595 and cefepime (FEP) showed a dose-dependent effect in a neutropenic murine thigh

74 infection model.(9) However, the effect of the combination therapy against severe
75 infections, such as pneumonia or bacteraemia, remains unknown.
76 In this study, we investigated the efficacy and pharmacokinetics of the
77 combination therapy of OP0595 and FEP in a mouse model of pneumonia caused by
78 ESBL-producing *K. pneumoniae.*
79

Materials and Methods

Bacterial strains

The *K. pneumoniae* strain used in this study was the KEN-11 strain, a clinical isolate obtained at the Nagasaki University Hospital.(10) The KEN-11 strain is positive for SHV and CTX-M-9-type ESBLs and showed positive results in the string test.(10) The bacteria were stored at -80°C in a Microbank® bead preservation system (Pro-Lab Diagnostics, Ontario, CA) until use.

Antimicrobial agents

OP0595 was supplied by Meiji Seika Pharma Co., Ltd. (Tokyo, Japan). Cefepime dihydrochloride hydrate was purchased from Bristol-Myers Squibb Company (Tokyo, Japan).

Animals

We purchased specific pathogen-free BALB/c male mice (6- to 7-week-old) from Japan SLC, Inc. (Shizuoka, Japan). The mice were housed in a pathogen-free environment and received sterile food and water at the Biomedical Research Centre of Nagasaki University.

Ethics

All the experimental protocols used in this study were approved by the Ethics Review Committee for Animal Experimentation (approval number 1503101199).

Antimicrobial susceptibility test

We tested the minimum inhibitory concentrations (MIC) of the agents against

105 the KEN-11 strain by a micro-dilution method with cation-adjusted Mueller-Hinton
106 broth (Becton, Dickinson & Co., Franklin Lakes, NJ) in accordance with the guidelines
107 of the Clinical and Laboratory Standards Institute.(11, 12) The final inoculum was
108 approximately 5×10^5 CFU/well. The MICs were defined as the lowest concentration
109 that inhibits visible growth after incubation at 35°C for 16 to 18 hours.
110
111 *Pharmacokinetic studies*
112 The Mice were infected with the bacterial suspension intra-tracheally (0.05
113 mL; 1×10^6 CFU/mouse). At 12 hours post inoculation, the mice were treated with 100
114 mg/kg of FEP and 20 or 100 mg/kg of OP0595, and were then sacrificed by cervical
115 dislocation at 5, 15, 30, 45, 60, 90, 120, and 180 minutes post administration. Blood
116 was collected via a right ventricular puncture using heparin-coated syringes. Four mice
117 were used for each group. The blood samples were centrifuged and the isolated plasma
118 samples were stored at -80°C until use.
119 The concentration of FEP and OP0595 in plasma was measured using a liquid
120 chromatograph (ACQUITY UPLC System, Waters, Milford, MA, United States)
121 coupled with a tandem mass spectrometer (QTRAP 5500, AB Sciex, Tokyo, Japan). For
122 pharmacokinetic analysis, WinNonlin Professional Ver. 6.3 (Certara) was used.
123 Plasma concentration was analysed using a non-compartmental model to
124 calculate the elimination half-life ($t_{1/2}$), apparent volume of distribution (V/F), area
125 under the plasma concentration-time curve from 0 to infinity ($AUC_{0\text{-inf}}$), and total body
126 clearance (CL).
127
128 *Preparation of bacteria and mouse model of pneumonia*

The method of preparation and inoculation of bacteria has been previously reported.(10) The KEN-11 strain was cultured overnight on a Muller-Hinton II agar (Becton Dickinson, Le Pont de Claix, France). To prepare the inoculum, a single colony of the bacteria was pre-incubated in Luria Bertani (LB) broth containing 100 μg/mL penicillin at 37°C for 18 hours with shaking at 250 rpm. After 8 hours of additional incubation in LB broth, the bacterial suspension was adjusted to appropriate concentrations using turbidimetry. The non-neutropenic mice were infected with the bacterial suspension intra-tracheally (0.05 mL; 1×10^6 CFU/mouse).

Treatment protocol

OP0595 and FEP were diluted with saline. Treatment commenced 12 hours post inoculation. Mice were intraperitoneally administered saline (control), 100 mg/kg of FEP, 20 mg/kg of OP0595, or FEP combined with OP0595, twice a day.

Bacteriological examinations

Mice were sacrificed from each group at 36 hours post inoculation. Subsequently, they were dissected under aseptic conditions. Blood was collected via a right ventricular puncture using heparin-coated syringes. The lungs were removed, suspended in 1 mL of normal saline, and homogenized with a homogenizer (AS One Co., Osaka, Japan). The lungs were then cultured and the serially diluted blood samples were spread onto the Mueller-Hinton II agar plates. After overnight incubation at 37°C, the number of visible colonies in the plates was evaluated. The lowest level of detectable bacterial count was 1×10^2 CFU/mL.

153 *Statistical analysis*

154 A statistical software package (StatMate V; ATMS Co., Ltd., Tokyo, Japan)
155 was used for all the statistical comparisons and the survival rates were calculated using
156 the Kaplan-Meier method. The survival analysis was performed using the log-rank test
157 and the data were expressed as the mean and standard deviation (SD). In the graph of
158 the bacterial count in the lungs and the blood, the data were depicted by a
159 box-and-whisker plot and the differences between the groups were analysed using a
160 one-way analysis of variance with Tukey's post-hoc test. All the tests of significance
161 were two-tailed and the alpha level for denoting statistical significance was set at $<$
162 0.05.

163

164

Results

MICs of the antimicrobial agents against KEN-11

The MICs of FEP and OP0595 against the KEN-11 strain were 8 and 4 mg/L, respectively. The MIC of FEP against the bacterial strain markedly improved in combination with OP0595 (Table 1).

Pharmacokinetics of the antimicrobial agents

The plasma concentration profiles and calculated pharmacokinetic parameters of FEP and OP0595 are presented in Fig. 1 and Table 2. The concentration of OP0595 in plasma with 20 mg/kg of OP0595 was significantly lower than that with 100 mg/kg of OP0595 at 5, 15, 30, 45, 60 and 180 minutes post treatment ($P < 0.05$) (Fig. 1). The areas under the plasma concentration-time curve from 0 to infinity ($AUC_{0\text{-inf}}$) of FEP in combination with 20 and 100 mg/kg of OP0595 were 93.89 and 82.18 μg·h/mL, respectively. The half-life ($t_{1/2}$) of FEP in combination with 20 and 100 mg/kg of OP0595 were 0.31 and 0.44 hours, respectively. Based on the MICs and pharmacokinetic parameters of FEP and OP0595, the percentage of time above MIC (%TAM) was calculated. The %TAM of FEP was increased by dose escalation of OP0595 (Fig. 2). The %TAM of 4, 20, and 100 mg/kg of FEP in combination with 20 mg/kg of OP0595 administered twice a day were 27.1, 27.7 and 27.7%, respectively.

Therapeutic effects of the antimicrobial agents on survival rate

Based on the results of pharmacokinetic studies, we decided the doses of OP0595 and FEP. Since we mainly investigated the effect of OP0595, such as inhibition of beta-lactamases and enhance effect of the antimicrobial activity of the beta-lactams that bind to PBP3, the mice were administered saline (control), 100 mg/kg of FEP, 20

189 mg/kg of OP0595, or FEP combined with OP0595, twice a day.

190 In the survival study, the mice were treated using the prescribed methods until
191 108 hours post inoculation and the survival rates were observed until 120 hours post
192 inoculation (n = 7 in each group). As shown in Fig. 3A, the survival rates were
193 significantly higher in the combination treatment group than that in the other groups (P
194 < 0.001).

195 In the bacteriological examinations, the mice were sacrificed 36 hours post
196 inoculation (n = 6 in each group). The number of bacteria in the lungs in the control,
197 OP0595, FEP, and combination treatment groups was 8.65 ± 0.58, 8.35 ± 0.40, 8.94 ±
198 0.48, and 4.47 ± 0.99 $\log_{10}$ CFU/mL, respectively (Fig. 3B). The number of bacteria in
199 the lungs was significantly lower in the combination treatment group than that in the
200 other groups (P < 0.001). The number of bacteria in the blood in the control, OP0595,
201 FEP, and combination treatment groups was 7.41 ± 0.93, 6.44 ± 0.71, 7.07 ± 1.20, and
202 4.49 ± 1.29 $\log_{10}$ CFU/mL, respectively (Fig. 3C). The number of bacteria in the blood
203 was significantly lower in the combination treatment group than that in the other groups
204 (P < 0.001 versus the control; P < 0.05, versus the OP0595 treatment group; P < 0.01,
205 versus the FEP treatment group).

206

207 *Effects of the combination therapy that depended on the doses of FEP*

208 Twelve hours post inoculation, the mice were treated twice a day with saline
209 (control), or 4, 20, or 100 mg/kg of FEP in combination with 20 mg/kg of OP0595 until
210 108 hours post inoculation. In the survival study, the survival rates were observed until
211 120 hours post inoculation (n = 9 in each group). As shown in Fig. 4A, the survival rates
212 were significantly higher in the 100 mg/kg of FEP treatment group than that in the other
213 groups (P < 0.001). The survival rates in the 4 and 20 mg/kg of FEP treatment groups

were significantly higher than that in the control group ($P < 0.05$, 4 mg/kg of FEP and $P < 0.01$, 20 mg/kg).

In the bacteriological examinations, the mice were sacrificed 36 hours post inoculation ($n = 4$, in each groups). The number of bacteria in the lungs in the control group and the 4, 20, and 100 mg/kg of FEP with OP0595 treatment groups was 9.42 ± 0.35, 7.46 ± 0.24, 6.53 ± 0.80, and 4.38 ± 0.69 $\log_{10}$ CFU/mL, respectively (Fig. 4B). The number of bacteria in the lungs was significantly lower in the 100 mg/kg of FEP with OP0595 treatment group than that in the other groups ($P < 0.001$). The number of bacteria in the lungs was significantly lower in the 4 and 20 mg/kg of FEP with OP0595 treatment groups than that in the control group ($P < 0.001$). The number of bacteria in the blood in the control, 4, 20, and 100 mg/kg of FEP with OP0595 was 8.14 ± 0.42, 7.17 ± 0.65, 5.46 ± 1.05, and 2.96 ± 0.68 $\log_{10}$ CFU/mL, respectively (Fig. 4C). The number of bacteria in the blood was significantly lower in the 100 mg/kg of FEP with OP0595 treatment group, than that in the other groups ($P < 0.001$). The number of bacteria in the blood was significantly lower in the 20 mg/kg of FEP with OP0595 treatment group than that in the control group ($P < 0.001$).

Discussion

The combination therapy of OP0595 and FEP showed good in vivo activity against ESBL-producing *K. pneumoniae* in the mouse model. *K. pneumoniae* often colonizes at the human digestive tract and nasopharynx,(13) and has been one of the major causes of both community and nosocomial pneumonia.(14) When bacteraemia is complicated with pneumonia, the mortality rate of patients with *K. pneumoniae* infection is 2-fold higher than that of patients with *S. pnuemoniae* infection.(15) In addition, the proportion of drug-resistant *K. pneumoniae* ESBL-producers is relatively high, and the use of appropriate therapy is independently associated with lower mortality.(2) Therefore, there is a need to develop a novel agent including OP0595 against ESBL-producing *K. pneumoniae.*

In the antimicrobial susceptibility test, the MIC of OP0595 against the KEN-11 strain was 4 mg/L, which was lower than that of FEP. A previous study reported that OP0595 directly inhibits the growth of many Enterobacteriaceae strains at concentrations of 1-8 mg/L by inhibiting PBP2, and in some strains, the MIC of OP0595 was lower than that of beta-lactams, such as piperacillin, FEP, and ceftazidime.(8) However, in the mouse model of pneumonia caused by *K. pneumonia*, the mice were treated with 20 mg/kg of OP0595, and monotherapy of OP0595 did not improve survival or decrease the number of bacteria in the lung and blood (Fig. 3). Hence, we consider that the effect of OP0595 in combination therapy did not depend on the direct antimicrobial activity of OP0595 against the KEN-11 strain.

OP0595 is a novel diazobicyclooctane that, similar to avibactam, inhibits class A and C serine beta-lactamases. In the previous study, the IC_{50} values of OP0595 for the class A and C beta-lactamases were similar to or slight higher than those of avibactam.(8) OP0595 also improved the MICs of beta-lactams in a dose-dependent

manner like avibactam does. (7–9) In this study, the MICs of FEP against KEN-11 markedly improved in combination with 0.5, 1.0, and 2.0 mg/L of OP0595 (Table 1). Additionally, in the mouse model, the effects of combination therapy of OP0595 and FEP depended on the dose of FEP (Fig. 4). From these results, the function of OP0595 in the combination therapy seems to be as a beta-lactamase inhibitor.

However, this does not explain the dose-dependent in vivo effects since the %TAM of 4, 20 and 100 mg/kg of FEP in combination with 20 mg/kg of OP0595 were almost the same (Fig. 2). If the in vivo effect depended on the inhibitory activity of OP0595 against beta-lactamase, then, the %TAM of FEP would increase according to the FEP dose. Moreover, the %TAM of FEP in combination with OP0595 was much lower than the effective %TAM in the previous study.(16, 17) Previous studies on OP0595 revealed that OP0595 enhances the activity of beta-lactams that bind to other PBPs besides PBP2.(7–9) In a neutropenic mouse model of thigh infection, the number of bacteria decreased in an FEP dose-dependent manner in combination with OP0595, and the action of OP0595 in the combination therapy was considered to be as a beta-lactamase inhibitor and beta-lactam enhancer.(9) In our mouse model of pneumonia as well, OP0595 in the combination therapy might act as both a beta-lactamase inhibitor and a beta-lactam enhancer.

There are concerns that the low dose of OP0595 will lead to the development of OP0595-resistant pathogens. A previous study revealed that sub-inhibitory concentrations of OP0595 caused resistance to OP0595 via activation of RpoS.(18) In addition, natural OP0595-resistant strains were reported.(8, 9) However, OP0595 showed a synergistic effect with beta-lactamases including FEP against OP0595-resistant strains via inhibition of beta-lactamases and enhancement of the activity of beta-lactams.(8, 9) Since the OP0595-resistant mutants caused small

282 increases of MICs of beta-lactams, (8, 9) further investigation about the
283 OP0595-resistance is needed.

284 This study has some limitations. First, only one clinical strain of *K.*
285 *pneumoniae* harbouring SHV- and CTX-M-9-type ESBLs was used in this study. The
286 activities of beta-lactams against ESBL-producers vary according to ESBL subgroups,
287 such as TEM-, SHV- and CTX-M-type.(19) In previous studies, OP0595 showed good
288 in vitro activity against various kinds of ESBLs, (7–9) but the in vivo efficacy against
289 ESBLs other than SHV and CTX-M-type remains unknown. Second, there was no
290 comparison of the combination therapy of OP0595 and FEP with the other novel
291 combination therapies, such as ceftazidime-avibactam and ceftolozane-tazobactam.
292 Third, it is unverifiable whether the combination therapy shows the same effect in
293 human, because there is no pharmacokinetic data in human. Finally, the toxicity of
294 OP0595 was not investigated in this study.

295 In conclusion, the results of our study demonstrated the in vivo effects of the
296 combination of OP0595 and FEP. Further investigations, including clinical trials, are
297 needed to study the effect of the combination therapy in patients with pneumonia caused
298 by ESBL-producing *K. pneumoniae*.

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Transparency declarations

None to declare

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Table 1
The MICs of FEP in combination with OP0595 against the KEN-11

Concentration of OP0595 (mg/L)	0	0.5	1.0	2.0
MIC of FEP (mg/L)	8	0.06	0.03	0.015

FEP, cefepime

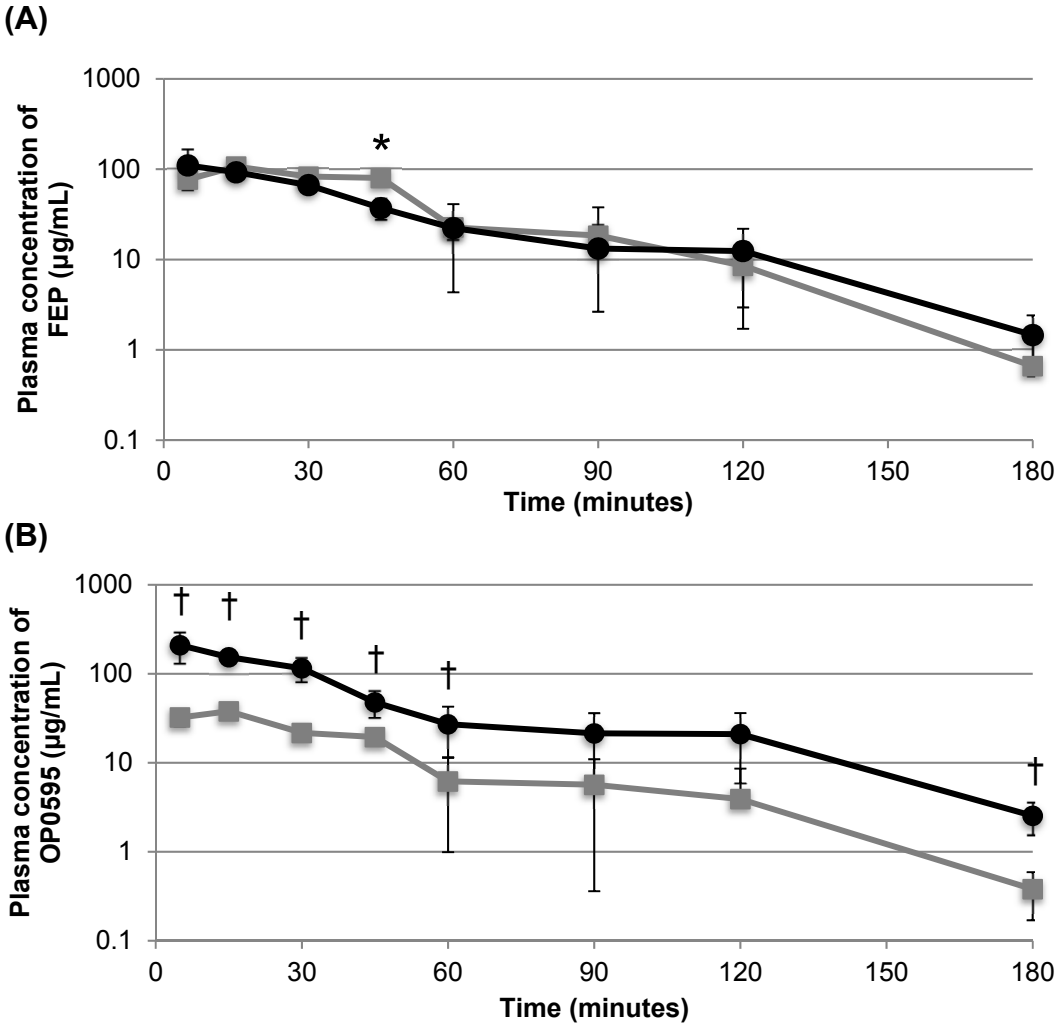

Fig. 1. Plasma concentrations of FEP and OP0595
The mice were treated with 100 mg/kg of FEP and 20 (filled grey squares) or 100 mg/kg (filled black circles) of OP0595 (n = 4 in each group). The pharmacokinetics of FEP (A) and OP0595 (B)was measured at 5, 15, 30, 45, 60, 90, 120 and 180 minutes. *The concentration of FEP in 100 mg/kg of OP0595 was significantly lower than that in 20 mg/kg of OP0595 at 45 minutes ($p < 0.05$). †The concentration of OP0595 in 100 mg/kg of OP0595 was significantly higher than that in 20 mg/kg of OP0595 at 5, 15, 30, 45, 60, and 180 minutes ($p < 0.05$).
FEP, cefepime.

Table 2
Selected pharmacokinetic parameters estimated for FEP and OP0595 in plasma

compound	Dose (mg/kg)	Combined drug	CL (L/h/kg)	V/F L/kg	$t_{1/2}$ (h)	$AUC_{0\text{-}inf}$ μg h/mL
FEP	100	20 mg/kg of OP0595	1.07	0.47	0.31	93.89
	100	100 mg/kg of OP0595	1.22	0.78	0.44	82.18
OP0595	20	100 mg/kg of FEP	0.68	0.36	0.37	29.55
	100	100 mg/kg of FEP	0.75	0.49	0.46	133.42

FEP, cefepime; CL, clearance; V/F, apparent volume of distribution; $t_{1/2}$, half-life; $AUC_{0\text{-}inf}$, area under the concentration-time curve from 0 to infinity.

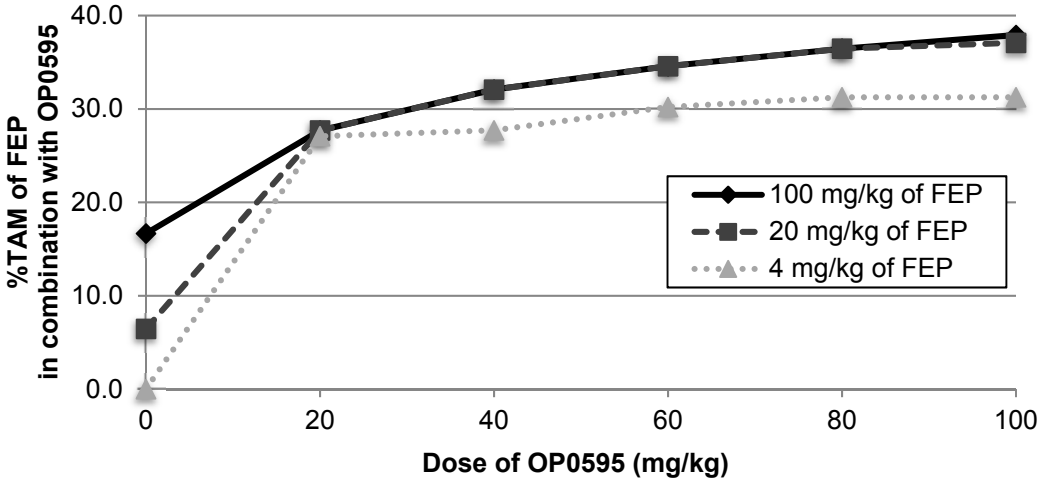

Fig. 2. %TAM of FEP in combination with OP0595
Based on the pharmacokinetic analysis, we calculated the percentage of time above MIC (%TAM) of FEP in combination with OP0595 against the bacteria. The %TAM of FEP twice a day was increased by dose escalation of OP0595 twice a day.
MIC, minimum inhibitory concentration; FEP, cefepime

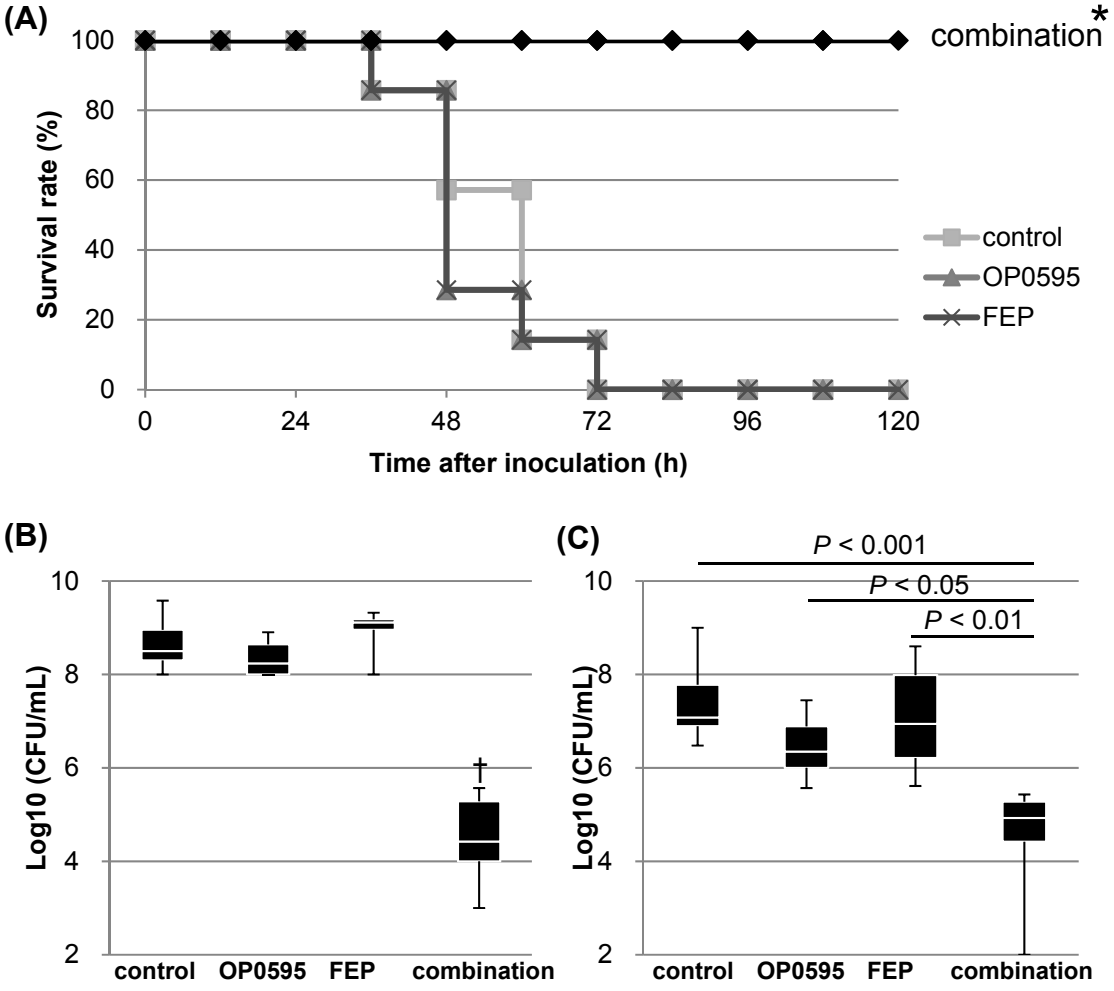

Fig. 3. Therapeutic effects of FEP in combination with OP0595.
Twelve hours post inoculation, the mice were treated twice a day with saline (control), 20 mg/kg of OP0595, 100 mg/kg of FEP or 100 mg/kg of FEP in combination with 20 mg/kg of OP0595 (combination) until 108 hours post inoculation. (A) The survival rates were observed until 120 hours after inoculation (n = 7 in each group). * The survival rate in the combination treatment group was significantly higher than the other treatment groups (P < 0.001). Thirty-six hours post inoculation, the mice were sacrificed, and the number of bacteria in the lungs (B) and the blood (C) were analysed (n = 6 in each groups). Box-and-whisker plots show the range and median of the number of bacteria. The number of bacteria in the lungs significantly decreased in the combination therapy group, compared with the other groups (P < 0.001). The number of bacteria in the blood significantly decreased in the combination therapy group, compared with the other groups (P < 0.001, versus the control; P < 0.05, versus the OP0595 treatment group; P < 0.01, versus the FEP treatment group).

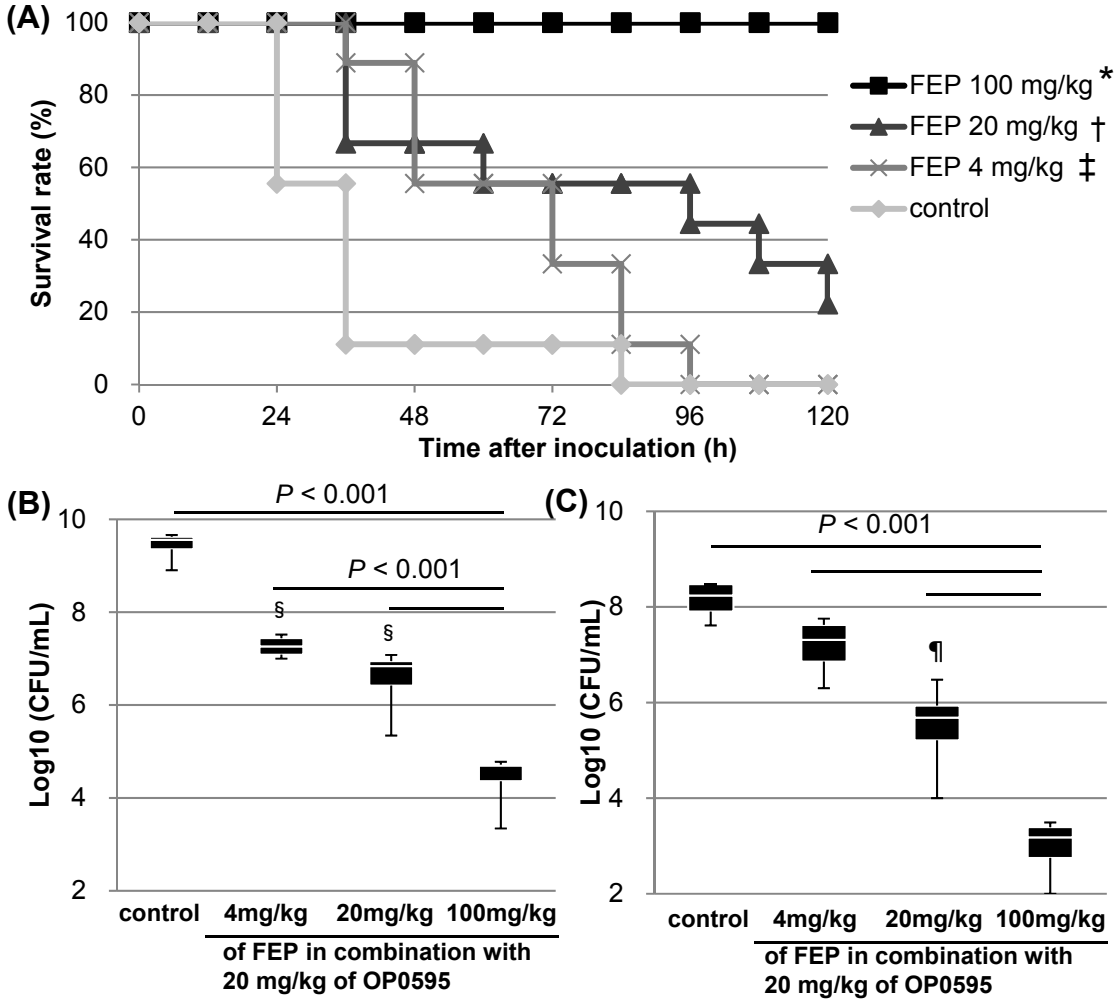

Fig. 4. Effects of combination therapy that depended on the doses of FEP
Twelve hours post inoculation, the mice were treated twice a day with saline (control), 4, 20, 100 mg/kg of FEP in combination with 20 mg/kg of OP0595 until 108 hours post inoculation. (A) The survival rates were observed until 120 hours after inoculation (n = 9 in each group). The survival rate in the 100 mg/kg of FEP treatment group was significantly higher than the other treatment groups (*P < 0.001). The survival rates in the 4 and 20 mg/kg of FEP treatment group were significantly higher than that in the control (†P < 0.05, 4 mg/kg of FEP and P < 0.01, 20 mg/kg of FEP, respectively). Thirty-six hours post inoculation, the mice were sacrificed, and the number of bacteria in the lungs (B) and the blood (C) were analysed (n = 4 in each groups). The number of bacteria in the lungs and blood were significantly lower in the 100 mg/kg of FEP, compared with the other groups (P < 0.001). The number of bacteria in the lungs was significantly decreased in the 4 and 20 mg/kg treatment group, compared with the control (§P < 0.001). The number of bacteria in the blood was significantly lower in the 20 mg/kg of FEP treatment group compared with the control (¶P < 0.001).