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Original Article

Pharmacokinetic and pharmacodynamic evaluation of sulbactam against *Acinetobacter baumannii* in *in vitro* and murine thigh and lung infection models.

Yuta Yokoyama[a], Kazuaki Matsumoto[a], Kazuro Ikawa[b], Erika Watanabe[a], Akari Shigemi[a], Yasuhiro Umezaki[a], Koyo Nakamura[a], Keiichiro Ueno[a], Norifumi Morikawa[b], Yasuo Takeda[a]*

[a]Department of Clinical Pharmacy and Pharmacology, Graduate School of Medical and Dental Sciences, Kagoshima University, 8-35-1 Sakuragaoka, Kagoshima 890-8520, Japan

[b]Department of Clinical Pharmacotherapy, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan

*Corresponding author

Tel.: +81-99-275-5543; Fax: +81-99-265-5293

E-mail: takeda@m.kufm.kagoshima-u.ac.jp

Abstract

Acinetobacter baumannii (*A. baumannii*) is a pathogen that has become globally associated with nosocomial infections. Sulbactam, a potent inhibitor of β-lactamases, was previously shown to be active against *A. baumannii* strains *in vitro* and effective against *A. baumannii* infections. However, a pharmacokinetic (PK)-pharmacodynamic (PD) analysis of sulbactam against *A. baumannii* infections has not yet been performed. This is necessary because optimization of dosing regimens should be based on PK-PD analysis. Therefore, we performed *in vitro* and *in vivo* PK-PD analyses of sulbactam using murine thigh and lung infection models of *A. baumannii* to evaluate the PK-PD of sulbactam. Sulbactam showed time-dependent bactericidal activity *in vitro* against *A. baumannii*. The PK-PD index that correlated best with its *in vivo* effects was the time that the free drug concentration remained above the MIC (*f*T > MIC) in both the thigh (R^2 = 0.95) and lung (R^2 = 0.96) infection models. The values of *f*T > MIC for a static effect, 1-, 2-, and 3-$\log_{10}$ kill were 21.0%, 32.9%, 43.6%, and 57.3% in the thigh infection model, and 20.4%, 24.5%, 29.3%, and 37.3% in the lung infection model, respectively. We reported the *in vitro* and *in vivo* time-dependent activities of sulbactam against *A. baumannii* infection, and demonstrated that sulbactam was sufficiently bactericidal when a *f*T > MIC of more than 60% against *A. baumannii* thigh infection and 40% against *A. baumannii* lung infection was achieved.

Keywords: sulbactam, *acinetobacter baumannii*, pharmacokinetic-pharmacodynamic, infection mouse model

1. Introduction

Acinetobacter baumannii is a significant global nosocomial pathogen [1] that has been associated with hospital-acquired infections including pneumonia, surgical site infection, urinary tract infections, and blood stream infections [2]. Although carbapenems are recommended as a first-line therapy, the prevalence of carbapenem-resistant Gram-negative bacteria is increasing, which has been attributed to the increased use of carbapenems [3, 4]. Therefore, an alternative to carbapenems is needed. Sulbactam, a potent inhibitor of β-lactamases, was previously shown to be active against *A. baumannii* strains *in vitro* and effective against *A. baumannii* infections [3, 5]. Fishbain et al. recommend at least 6 g per day in divided doses for patients with normal renal function, but the optimal dosing of sulbactam to treat serious *A. baumannii* infections is unknown [6]. At present, pharmacokinetic (PK)-pharmacodynamic (PD) analysis is gaining much attention with promising results as it is able to optimize the dosing regimen, thereby improving outcomes [7]. PK-PD analyses, based on the principle reported by Craig [8], and other researchers to optimize dose regimens for clinical application, are now increasing in the United States and Europe. However, a PK-PD analysis of sulbactam against *A. baumannii* infections has not yet been performed, optimized dosing regimens based on PK-PD analysis is necessary. The aims of our study were to evaluate the *in vitro* antimicrobial effect of sulbactam and determine the PK-PD index using murine thigh infection and lung infection models of *A. baumannii*.

2. Materials and methods

2.1. Bacterial strains and media

A. baumannii was used in this study: reference strain ATCC 19606 (ATCC, Rockford, MD). A strain was stored in the Microbank (Iwaki Co. Ltd., Tokyo, Japan) at -80°C. Prior to each experiment, strains were subcultured on sheep blood agar (NISSUI PHARMACEUTICAL Co. Ltd., Tokyo, Japan) and incubated at 37°C.

2.2. Antibiotics

Sulbactam sodium salt was purchased from Funakoshi Co. Ltd. (Tokyo, Japan). This agent was used as a standard laboratory powder in the *in vitro* and *in vivo* studies.

2.3. Antimicrobial Susceptibility Testing

The minimum inhibitory concentration (MIC) for sulbactam was determined using the standardized agar dilution method according to Clinical and Laboratory Standards Institute guidelines [9]. A suspension of bacteria equivalent to the 0.5 McFarland turbidity standards was inoculated onto Mueller-Hinton agar (MHA) plates. An E-test for sulbactam was plated onto the agar. The MIC value was read following 16-20 h of incubation at 37°C. *A. baumannii* ATCC 19606 was used as the quality

control strain.

2.4. Time-kill curve

Experiments were performed in tubes with 2 mL MHB. Bacteria from a 4-h logarithmic-growth-phase culture were added to obtain a start inoculum of 10^6 CFU/mL. The bacterial inoculum and no antimicrobial drug were used as growth controls. Time-kill curves were performed using sulbactam concentrations of one-fourth, 1, 4, 16, and 64 × the MIC, and bacterial growth was quantified after 0, 2, 4, 6, and 8 h of incubation at 37°C. Ten-fold dilutions were spread onto MHA and cultured at 37°C for 24 h.

2.5. In vivo studies

Neutropenic murine thigh and lung infection models

The use of animals in the present study was approved by the Institutional Animal Care and Use Committee of Kagoshima University (approval number: MD12011). Neutropenic murine thigh and lung infection models were described previously by Dudhani et al [10, 11]. Five-week-old female ddY mice were rendered neutropenic by injecting cyclophosphamide intraperitoneally (i.p.) 4 days (150 mg/kg) and 1 day (100 mg/kg) prior to experimental infection. Mice were anesthetized with an i.p. injection of 0.3 mg/kg of medetomidine, 4.0 mg/kg of midazolam, and 5.0 mg/kg of

butorpanol before the bacterial inoculation [12]. Thigh infection was produced by injecting 100 μL of an early-logarithmic-phase bacterial suspension (3.75×10^6 CFU/mL) intramuscularly into one posterior thigh muscle. Lung infection was produced by intranasally introducing 50 μL of a final inoculum of 7.5×10^6 CFU/mL mixed 1:1 with 6% porcine mucin of bacterial cells (3.75×10^6 CFU/mL) in the early logarithmic phase. Thereafter, animals were held in a vertical position with their head up for 1 min. The sulbactam treatment commenced 2 h after the inoculation in both models, by which time an infection was reproducibly established.

2.6. Serum concentration of sulbactam

Single-dose serum PK studies of sulbactam were performed in neutropenic mice after the subcutaneous administration of sulbactam (30, 60, 120, and 240 mg/kg). Blood samples (1.0 mL) were obtained 5, 15, 30, 60, and 90 min after the subcutaneous administration, followed by cervical dislocation prior to intracardiac puncture (three animals per time point). The sulbactam concentration of each sample was determined by a high-performance liquid chromatography (HPLC) method [13], with minor modifications.

2.7. Serum protein binding of sulbactam

Protein binding studies were conducted using centrifugal filter units [14]. Serum samples were incubated for thirty minutes at 37°C, placed into the prewarmed centrifuge chamber, and were then spun at 2,000 × g for 10 minutes. Percentage protein binding (%PB) at each prepared concentration was calculated using the following equation: %PB = [(S-SUF)/S] × 100, where S is the sulbactam concentration in the

initial serum solutions and SUF is the sulbactam concentration in the ultrafiltrate. Sulbactam concentrations in the serum and ultrafiltrate were measured using HPLC.

2.8. Pharmacodynamics of sulbactam in neutropenic mouse thigh and lung infection models

The sulbactam treatment was initiated 2 h following the bacterial inoculation in the thigh muscle and lung infection studies. Sulbactam regimens for thigh-infected animals involved subcutaneous doses over a range of 15-240 mg/kg and were administered at 2, 3, 4, 6, 12, and 24 h intervals with 18 dosing patterns. Each dosing regimen involved three mice. Sulbactam-treated mice were humanely killed and their thighs were removed 24 h after the initiation of treatment. Samples were collected aseptically from the untreated control group 2 and 26 h after the bacterial inoculation to count the number of viable cells. The removed thighs were individually homogenized in 7 mL of sterile normal saline. The thigh homogenate was serially diluted ten-fold (six serial dilutions) with MHB, and 50 μL each of the diluents were spread onto BTB agar and cultured at 37°C for 24 h. The number of CFU was counted for each thigh and expressed as the number of $\log_{10}$ CFU per thigh. The lower limit of counting was 160 CFU per thigh. Sulbactam regimens for lung-infected animals were as described above with 18 dosing patterns. Each dosing regimen involved three mice. Animals were humanely killed 2 h (untreated controls) and 24 h (untreated controls plus sulbactam-treated mice) after the inoculation. The lungs were collected aseptically and individually homogenized in 1.7 mL of sterile normal saline. The counts of viable bacteria in the right and left lungs were determined as described above. The lower limit of counting was 130 CFU per lung.

150

151 *2.9. PK-PD analyses of sulbactam*

152 Drug concentration data for each dose of 30, 60, 120, and 240 mg/kg were
153 fitted to a standard one-compartment model with first order absorption and elimination
154 processes. The pharmacokinetic parameters in this model were apparent volume of
155 distribution (Vd), absorption rate constant (*ka*), and elimination rate constant (*ke*).

156 Using the mean pharmacokinetic parameters (Vd, *ka* and *ke*) for the four doses,
157 serum sulbactam concentrations were then simulated to estimate three major PK-PD
158 indices: the time that the free drug concentration remained above the MIC ($fT > MIC$),
159 the ratio of the area under the free concentration-time curve for a 24 h period to the MIC
160 ($fAUC_{24}/MIC$), and the ratio of the maximum free concentration to the MIC
161 (fC_{max}/MIC).

162 Data for antibacterial activities in the thigh and lung were fitted to a standard
163 sigmoid E_{max} model: $E = E_0 - (E_{max}*X^{\gamma})/(EC_{50}^{\gamma} + X^{\gamma})$, where E is the killing effect of
164 sulbactam (log_{10} CFU of the *A. baumannii* per thigh or lung at 24 h), E_0 is the baseline
165 effect in the absence of the drug, E_{max} is the maximum killing effect, X is the PK-PD
166 index ($fT > MIC$, $fAUC_{24}/MIC$ or fC_{max}/MIC), EC_{50} is the PK-PD index value needed
167 for 50% of E_{max}, and γ is the Hill coefficient describing the steepness of the sigmoid
168 curve.

169 These PK-PD analyses were performed with nonlinear least-squares regression by the
170 MULTI program [15].

171 Statistical analysis was performed using a Mann-Whitney test.

3. Results

3.1. Antimicrobial Susceptibility Testing

The MIC of sulbactam against *A. baumannii* ATCC 19606 was 0.5 μg/mL.

3.2. Time-kill curve

Figure 1 illustrates a series of time-kill curves for a standard strain of *A. baumannii* exposed to sulbactam at concentrations ranging from one-fourth to 64 times the MIC. The time-kill curve at the concentration of 4-64 times the MIC showed in time-dependently, but not in concentration dependently, to decrease bacterial cell number. The point of maximum effect occurred at about 4 times the MIC, so an increase in the rate on extent of killing was negligible once sulbactam concentrations exceed the MIC. Therefore, sulbactam exhibited time-dependent bactericidal activity against *A. baumannii*.

3.3. Sulbactam pharmacokinetics

The concentrations of sulbactam in neutropenic infection mice following single subcutaneous doses of 30, 60, 120, and 240 mg/kg are shown in Figure 2. The serum PK parameters are summarized in Table 1. The ranges of C_{max} and AUC_{24} were 23.36-230.76 μg/mL and 15.95-142.28 mg·h/L, respectively. The mean ± standard

deviation (SD) of Vd, *ke*, and *ka* for the four doses were 0.43 ± 0.02 L/kg, 4.01 ± 0.18 h^{-1}, and 4.27 ± 0.47 h^{-1}, respectively. Serum protein binding of sulbactam was 5.20 ± 1.25%.

3.4. Relationships between PK-PD indices and antibacterial effect

At the start of treatment (2 h after inoculation), the mean ± SD bacterial load in thigh-infected animals was 6.23 ± 0.18 $\log_{10}$ CFU/thigh. Bacterial numbers grew 0.83 ± 0.05 $\log_{10}$ CFU/thigh in untreated control mice over the next 24 h. The maximal reduction of CFU in sulbactam-treated animals after 24 h exposure was observed 4.86 ± 0.05 $\log_{10}$ CFU/thigh down compared to numbers at the start of treatment. The relationships between the antibacterial effect and each of the PK-PD indices (*f*T > MIC, *f*AUC_{24}/MIC and *f*C_{max}/MIC) for *A. baumannii* ATCC 19606 are shown in Figure 3. Regarding the PK-PD indices of sulbactam, the therapeutic efficacy of sulbactam correlated with *f*T > MIC (R^2 = 0.95) more than *f*AUC_{24}/MIC (R^2 = 0.60) or *f*C_{max}/MIC (R^2 = 0.37) in the thigh infection model. PK-PD model parameter estimates for the *f*T > MIC index for sulbactam against *A. baumannii* in the thigh infection model are shown in Table 2. The E_{max} was 5.19 $\log_{10}$ CFU/thigh down after 24 h exposure compared to numbers at the start of treatment. The E_0 was 0.52 $\log_{10}$ CFU/thigh. The bacterial

209 numbers grew after 24 h in untreated control mice. The EC_{50} was 44.6%.

210 At the start of treatment (2 h after the inoculation), the mean ± SD bacterial
211 load in lung-infected animals was 6.21 ± 0.20 log_{10} CFU/lung. Over the next 24 h,
212 bacterial numbers grew 2.37 ± 0.38 log_{10} CFU/lung in untreated control mice. The
213 maximal reduction of CFU in sulbactam-treated animals after 24 h exposure was
214 observed 6.44 ± 0.27 log_{10} CFU/thigh down compared to numbers at the start of
215 treatment. Relationships between the antibacterial effect and each of the PK-PD indices
216 (*f*T > MIC, *f*AUC_{24}/MIC and *f*C_{max}/MIC) for *A. baumannii* ATCC 19606 are shown in
217 Figure 4. Regarding the PK-PD indices of sulbactam, the therapeutic efficacy of
218 sulbactam correlated with *f*T > MIC (R^2 = 0.96) more than *f*AUC_{24}/MIC (R^2 = 0.68) or
219 *f*C_{max}/MIC (R^2 = 0.40) in the lung infection model. PK-PD model parameter estimates
220 for the *f*T > MIC index for sulbactam against *A. baumannii* in the lung infection model
221 are shown in Table 2. The E_{max} was 5.81 log_{10} CFU/lung down after 24 h exposure
222 compared to numbers at the start of treatment. The E_0 was 1.87 log_{10} CFU/lung. The
223 bacterial numbers grew after 24 h in untreated control mice. The EC_{50} was 24.7%.

224

225 *3.5. Magnitude of the PK-PD index associated with efficacy*

226 Table 3 shows the values of *f*T > MIC required for a static effect and 1-, 2-, and

227 3-$\log_{10}$ reductions in the bacterial burden. The values of *f*T > MIC for a static effect, 1-,

228 2-, and 3-$\log_{10}$ kill were 21.0%, 32.9%, 43.6%, and 57.3% in the thigh infection model,

229 and 20.4%, 24.5%, 29.3%, and 37.3% in the lung infection model, respectively. There

230 was little difference in the *f*T > MIC required to achieve a given magnitude of effect in

231 both models.

4. Discussion

The present study was designed to characterize the PK-PD characteristics of sulbactam against *A. baumannii*. Sulbactam has been shown to exhibit direct antimicrobial activity against *A. baumannii* [16], even though it is a potent inhibitor of β-lactamases. In the *in vitro* experiments, sulbactam exhibited bactericidal activity against *A. baumannii* (Fig. 1). The time-kill curve is a standard technique that is used to demonstrate the time course of bactericidal activity. Two major patterns of bactericidal activity were observed with increasing drug concentrations [17]. The first pattern was characterized by marked concentration-dependent killing over a wide range of concentrations. This pattern of killing has been observed with aminoglycosides and fluoroquinolones. The second pattern was characterized by saturation of the rate of killing at concentrations near the MIC. High concentrations did not kill the organism faster or more extensively than low concentrations. Therefore, the duration of exposure rather than the concentration was the major determinant of the extent of killing. As shown in Figure 1, sulbactam showed bactericidal activity called time-dependent killing as commonly as other β-lactams. In a murine pneumonia model using an imipenem- and sulbactam-susceptible *A. baumannii* isolate, similar efficacies were observed between these agents when the dosing of sulbactam reached a time above the MIC, similar to

250 that of imipenem, which confirmed the time-dependent activity of this antimicrobial
251 [18]. The T > MIC is known to be the most predictive PK-PD parameter of the *in vivo*
252 efficacy of β-lactams in animal models [19].

253 PK-PD analysis using a murine infection model has become a standard method
254 to predict clinical efficacy and is often used to determine optimal doses for clinical trials.
255 This method was established by Craig et al [19]. In the *in vivo* experiments, we used
256 two murine infection models to determine, for the first time, the PK-PD index most
257 predictive of the activity of sulbactam against *A. baumannii*, and also the magnitude of
258 the predictive index required for various magnitudes of the killing effect. Non-linearity
259 was a feature of the unbound PK of sulbactam in neutropenic mice. The PK nonlinearity
260 noted in this study was observed over the very wide range of sulbactam doses needed to
261 fully characterize the PK-PD relationship. The superposition principle was applied to
262 single-dose unbound plasma sulbactam concentration-time curves to generate the
263 unbound plasma concentration for various dosage regimens across the 24 h treatment
264 period. The *f*T > MIC ratio in the thigh and lung infection models appeared to be
265 slightly more predictive of *in vivo* bacterial killing than fC_{max}/MIC or $fAUC_{24}$/MIC
266 based on R^2 values and a visual examination of the fits (Fig. 3 and 4). The *f*T > MIC
267 targets required for a static effect against *A. baumannii* thigh and lung infection were

268 estimated to be approximately 20%, 1-, 2-, and 3-$\log_{10}$ kill were estimated as 32.9%,
269 43.6%, and 57.3% in the thigh infection model, and 24.5%, 29.3%, and 37.3% in the
270 lung infection model, respectively (Table 3). The *f*T > MIC required for a static effect in
271 the *A. baumannii* thigh and lung infection models were similar, the *f*T > MIC required
272 for 3-$\log_{10}$ kill in the thigh infection model was generally higher than that required in
273 the lung infection model. The activity of sulbactam was slightly more enhanced in the
274 lung than in the thigh. This may reflect differences in bacterial behavior between the
275 two sites and/or the somewhat restricted access of sulbactam to the infection site in the
276 thigh relative to the level of access to the infection site in the lung. Over the past 15
277 years, numerous PK-PD data has been good concordance between PK-PD animal
278 studies and data from infected patients [20]. In mice, the *f*AUC_{24}/MIC ratio of quinolons,
279 70-90 was associated with 2-$\log_{10}$ kill reduction in bacterial burden, which is very
280 similar to the *f*AUC_{24}/MIC breakpoint identified in infected patients (*f*AUC_{24}/MIC ratio,
281 ≥87). In this study, we estimated that achievement of animal derived PK-PD target was
282 the 3-$\log_{10}$ kill. The adjustment of higher dosing regimen performed to improve the
283 outcome for severe infection and immunocompromised patients. Therefore, the *f*T >
284 MIC targets required for a static effect against *A. baumannii* thigh and lung infection
285 were 20%. The *f*T > MIC targets required for the sufficient bactericidal effects against *A.*

286 *baumannii* thigh and lung infection were 60% and 40%, respectively. The *f*T > MIC
287 targets of β-lactam antibiotics (carbapenems, penicillins, and cephalosporins) required
288 for a static effect and near maximal bactericidal effects against organism were 20-40%
289 and 40-70%, respectively [8].

290 Lastly, the experimental design of this study was limited. We could not assess
291 with other bacterial isolates, other animals and observation times longer than 24 h.
292 Further studies are required to confirm our findings and clarify their clinical
293 implications.

294 In conclusion, two murine infection models were used to identify the PK-PD
295 index most predictive of the antibacterial activity of sulbactam against *A. baumannii*,
296 and the magnitude of the predictive index required for various magnitudes of the effect.
297 This study has defined the *f*T > MIC targets needed to achieve various magnitudes of
298 bacterial kill. We showed the *in vitro* and *in vivo* time-dependent activities of sulbactam
299 against *A. baumannii* infection, and demonstrated that sulbactam was sufficiently
300 bactericidal when a *f*T > MIC of more than 60% against *A. baumannii* thigh infection
301 and 40% against *A. baumannii* lung infection was achieved.

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307 *Competing interests*: None declared.

308 *Ethical approval*: This study was approved by the IACUC of Kagoshima University

309 (approval number: MD12011).

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389 **Figure legends**

390

391 **Fig. 1.** Time-kill curves of *A. baumannii* ATCC 19606 with exposure to sulbactam at

392 concentrations from one-fourth to 64 times the MIC (n = 3, mean ± SD).

393

394 **Fig. 2.** Pharmacokinetic profiles for single subcutaneous doses (mg/kg) of sulbactam in

395 neutropenic-infected mice (n = 3, mean ± SD). Simulation curves were generated using

396 Vd = 0.43 L/kg, ke = 4.01 h^{-1} and ka = 4.27 h^{-1}.

397

398 **Fig. 3.** Relationships for *A. baumannii* ATCC 19606 between the $\log_{10}$ CFU/thigh at 24

399 h and PK-PD indices (A) fT > MIC, (B) fAUC$_{24}$/MIC, and (C) fC$_{max}$/MIC. Each symbol

400 represents the mean ± SD for one thigh per mouse. The horizontal dashed lines

401 represent the organism burden at the start of the therapy. R^2 is the coefficient of

402 determination.

403

404 **Fig. 4.** Relationships for *A. baumannii* ATCC 19606 between the $\log_{10}$ CFU/lung at 24 h

405 and PK-PD indices (A) fT > MIC, (B) fAUC$_{24}$/MIC, and (C) fC$_{max}$/MIC. Each symbol

406 represents the mean ± SD for a single lung per mouse. The horizontal dashed lines

represent the organism burden at the start of the therapy. R^2 is the coefficient of determination.

Fig. 1

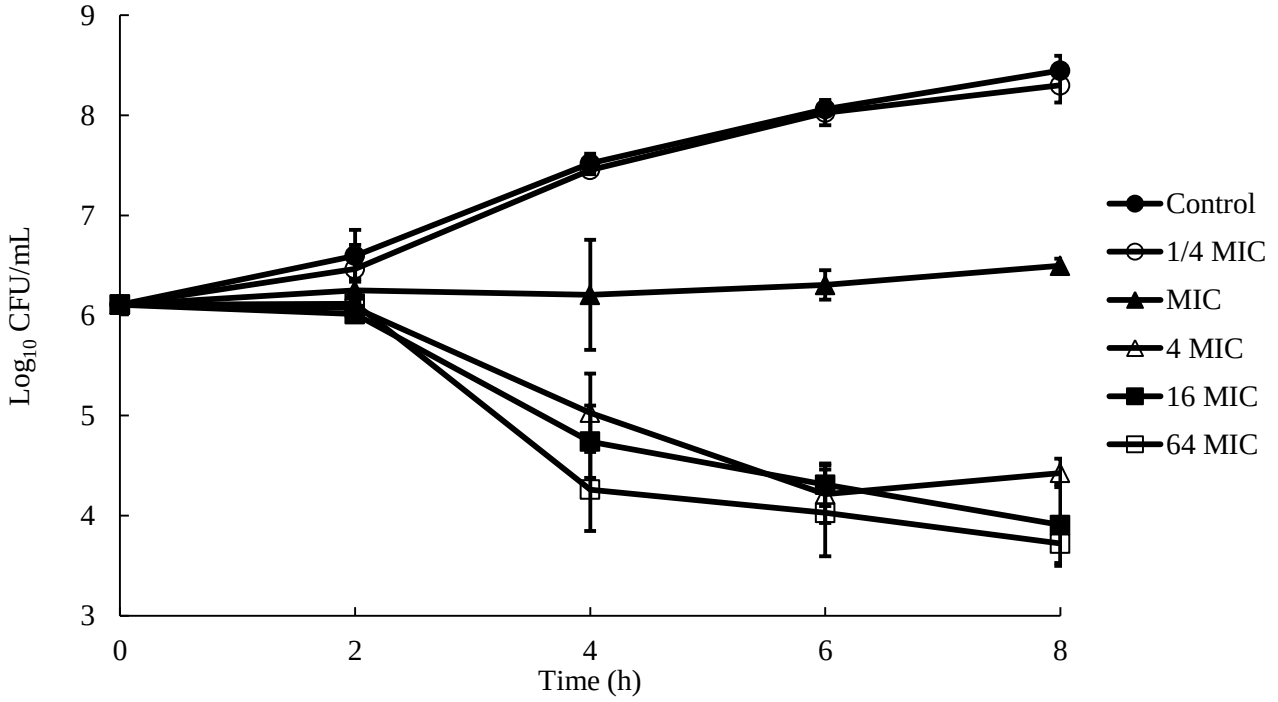

9
8
7
6
5
4
3
Log10 CFU/mL
0
2
4
6
8
Time (h)
Control
1/4 MIC
MIC
4 MIC
16 MIC
64 MIC

Fig. 2

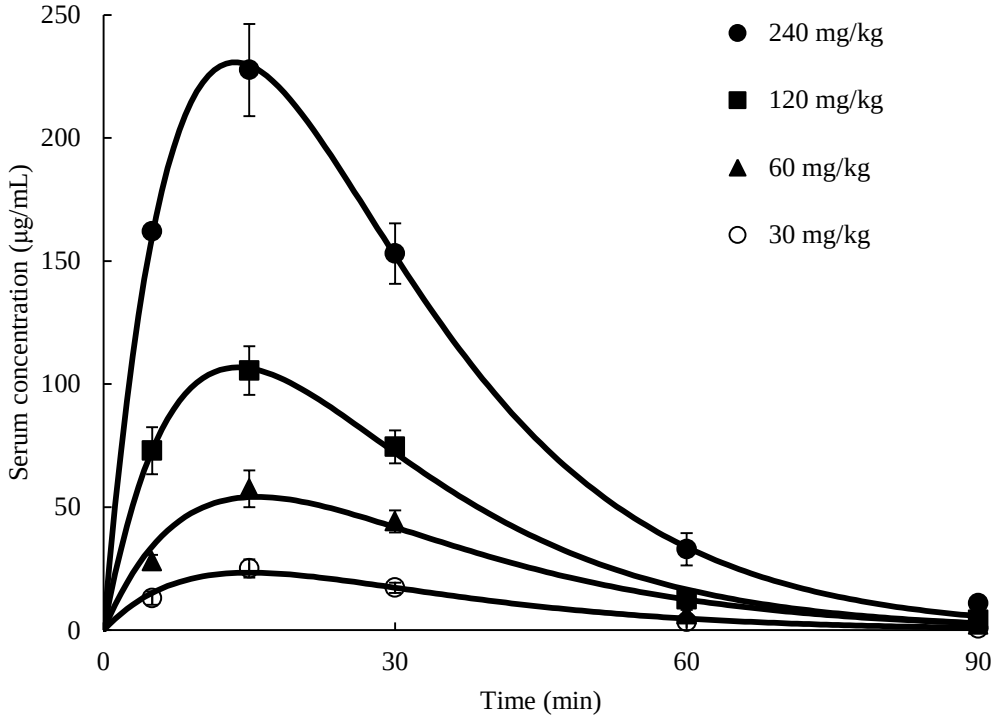
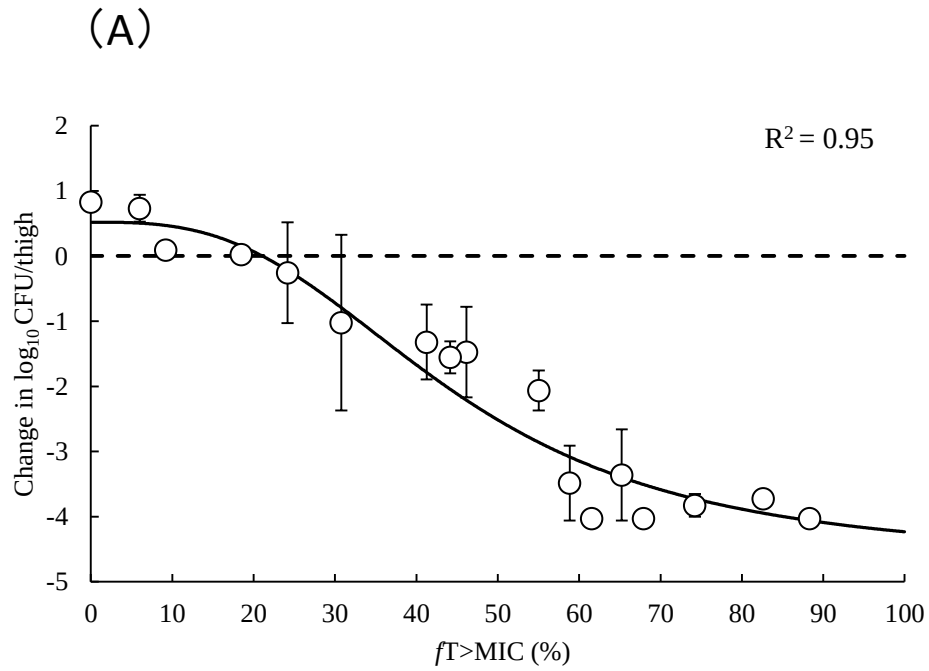
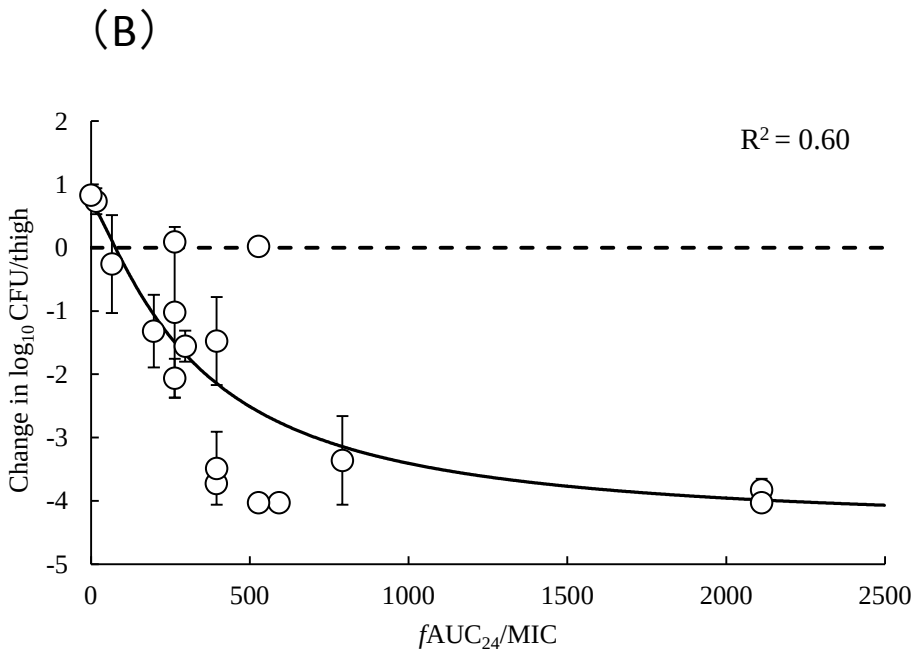

250
200
150
100
50
0
Serum concentration (μg/mL)
0
30
60
90
Time (min)
240 mg/kg
120 mg/kg
60 mg/kg
30 mg/kg

Fig. 3


(A)
R² = 0.95
Change in log10 CFU/thigh
fT>MIC (%)


(B)
R² = 0.60
Change in log10 CFU/thigh
fAUC24/MIC

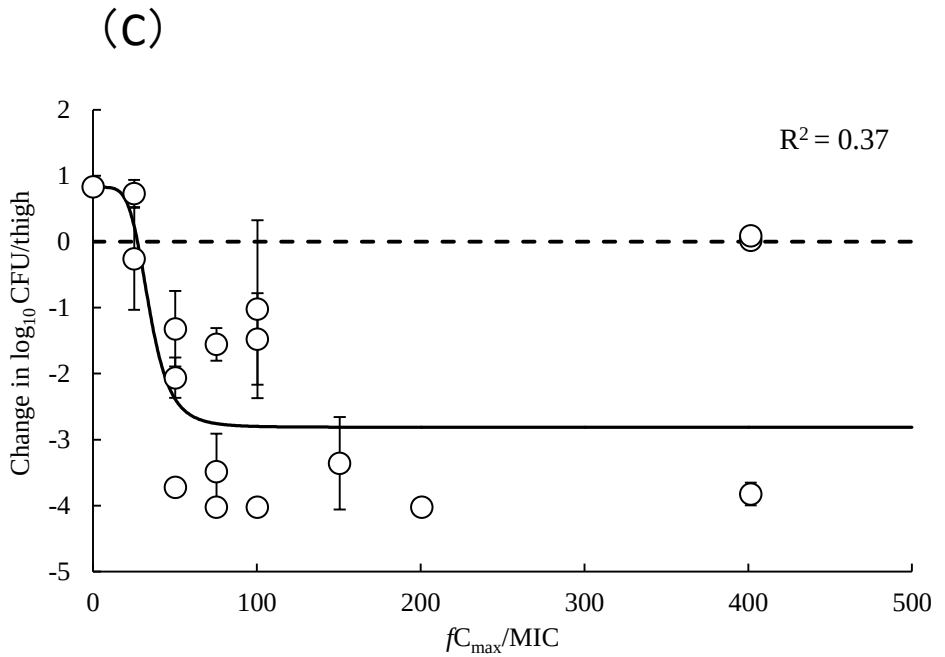
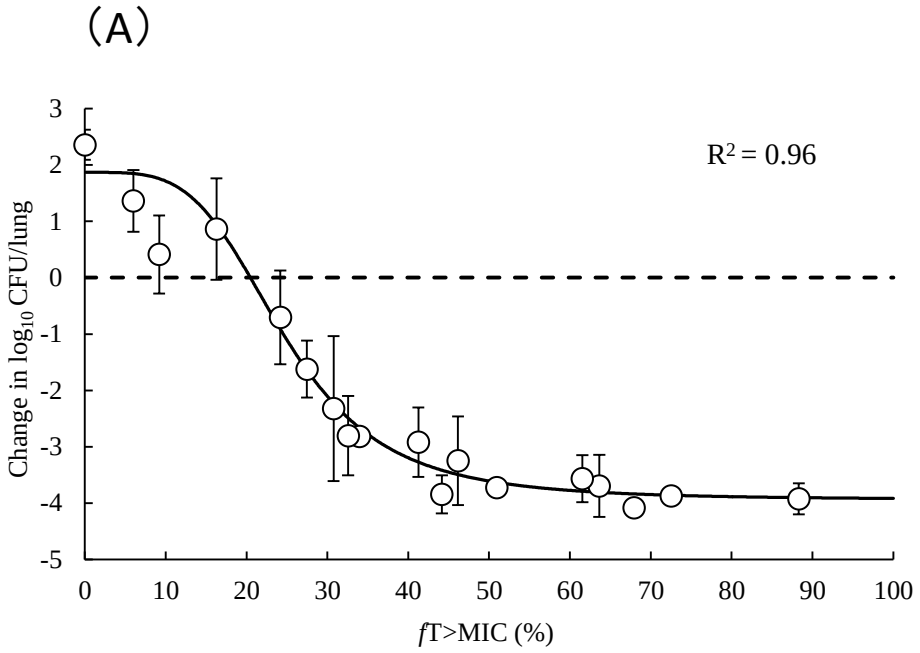
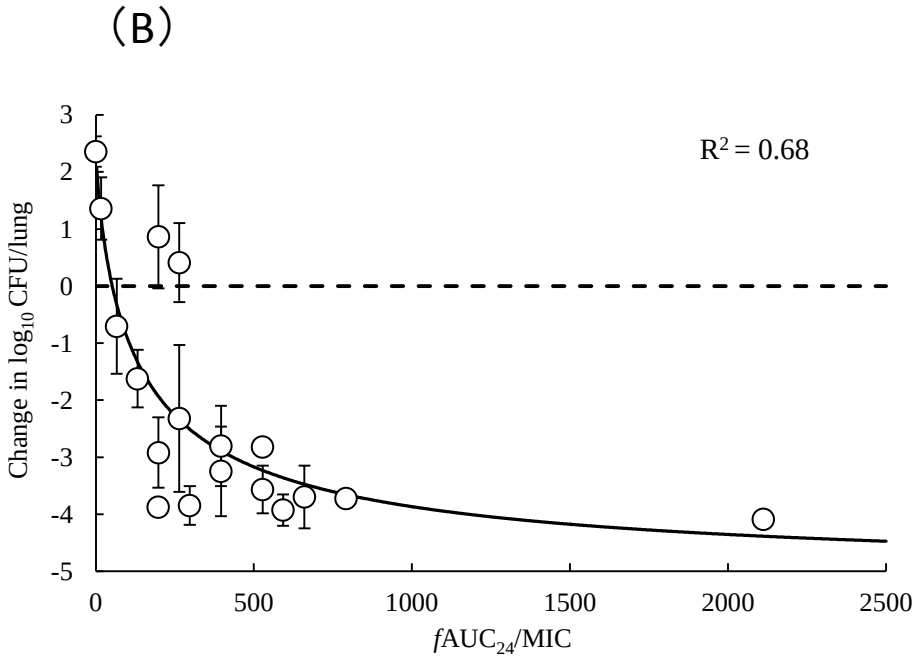

(C)
R² = 0.37
Change in log10 CFU/thigh
fCmax/MIC

Fig. 4


(A)
R² = 0.96
Change in log10 CFU/lung
fT>MIC (%)


(B)
R² = 0.68
Change in log10 CFU/lung
fAUC24/MIC

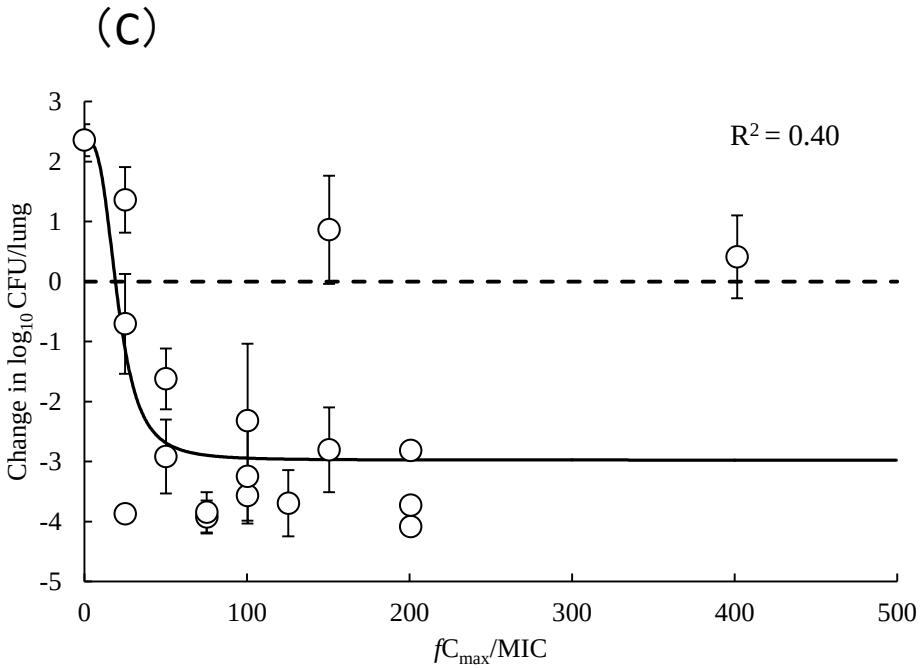

(C)
R² = 0.40
Change in log10 CFU/lung
fCmax/MIC

Table 1

Parmacokinetic parameters of sulbactam after a single subcutaneous

Dosing regimen (mg/kg)	C_{max} (μg/mL)	AUC_{24} (mg·h/L)
30	23.36	15.95
60	54.15	38.70
120	106.76	66.99
240	230.76	142.28

Table 2

PK-PD model parameter estimates predicting viable counts at 24 h for the *f*T > MIC index of sulbactam against *A. baumannii* in the thigh and lung infection models.

Model	E_{max} (log_{10} CFU/Organ)	E_0 (log_{10} CFU/Organ)	EC_{50} (%)	γ
Thigh infection	5.19	0.52	44.6	2.93
Lung infection	5.81	1.87	24.7	3.98

Table 3

Target values of sulbactam *f*T > MIC (%) for a static effect and 1-, 2-, and 3-$\log_{10}$ kill against *A. baumannii* in the thigh and lung infection models.

	Thigh infection	Lung infection
Static effect	21.0	20.4
1-$\log_{10}$ kill	32.9	24.5
2-$\log_{10}$ kill	43.6	29.3
3-$\log_{10}$ kill	57.3	37.3