Development of an Optimised Pharmacokinetics/Pharmacodynamics analysis method of β-lactam/nacubactam against Carbapenemase-Producing *K. pneumoniae*

Yuki Igarashi[1], Wataru Takemura[1], Xiaoxi Liu[1], Nana Kojima[1], Takumi Morita[1], Victor Chuang[2], Yuki Enoki[1], Kazuaki Taguchi[1], and Kazuaki Matsumoto[1]

[1]Keio University
[2]Curtin University Curtin Medical School

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Abstract

Background and Purpose: No pharmacokinetics/pharmacodynamics (PK/PD) analysis method has been established for combination therapy that comprehensively reflects the efficacy of both antibiotics. Recently, nacubactam, which is a DBO-type new β-lactamase inhibitor and has antibacterial activity, is being developed as a single drug to be co-administered with cefepime or aztreonam. This study attempted to establish a PK/PD analysis method for β-lactam/β-lactamase inhibitors that incorporates instantaneous MIC (MIC_i) to determine practical PK/PD parameters for aztreonam/nacubactam. **Experimental Approach:** Based on Checkerboard MIC measurements, MIC_i of aztreonam against carbapenemase-producing *Klebsiella pneumoniae* in the presence of nacubactam was simulated. *In vivo* PD effect was evaluated by the bacterial count of thigh-infected mice after administered a combination of nacubactam and aztreonam. The mean change in the bacterial count obtained by *in vivo* PD study was plotted based on $\%fT{>}MIC_i$ and analysed using the Inhibitory Effect Sigmoid I_{max} Model. **Key Results:** $fT{>}MIC_i$ calculated from the PK experiments showed a high correlation with the bactericidal effect obtained in the PD experiments, suggesting that $fT{>}MIC_i$ is the optimal PK/PD parameter for aztreonam/nacubactam. The target values of $fT{>}MIC_i$ achieving growth inhibition, 1 $\log_{10}$-kill and 2 $\log_{10}$-kill, were 22, 38 and 75%, respectively. **Conclusion and Implications:** The PK/PD analysis method proposed in this study is promising for determining practical PK/PD parameters in a combination antimicrobial therapy. In addition, this is the first report of aztreonam/nacubactam showing a potent *in vivo* therapeutic effect against carbapenemase-producing *K. pneumoniae*, particularly NDM-producing *K. pneumoniae*.

Τιτλε: Δεελοπμεντ οφ αν Οπτιμισεδ Πηαρμαχοκινετιχς/Πηαρμαχοδψναμιχς αναλψσις μετηοδ οφ β-λαχταμ/ναχυβαχταμ αγαινστ ἂρβαπενεμασε-Προδυχινγ *Κ. πνευμονιαε*

Authors : Yuki Igarashi[1], Wataru Takemura[1], Xiaoxi Liu[1], Nana Kojima[1], Takumi Morita[1], Victor Tuan Giam Chuang[2], Yuki Enoki[1], Kazuaki Taguchi[1,*], Kazuaki Matsumoto[1]

Institutional Affiliations : [1] Division of Pharmacodynamics, Keio University Faculty of Pharmacy, 1-5-30 Shibakoen, Minato-ku, Tokyo 105-8512, Japan

[2] Discipline of Pharmacy, Curtin Medical School, Faculty of Health Sciences, Curtin University, GPO Box U1987, Perth, Western Australia 6845, Australia.

*Corresponding author: Kazuaki Taguchi, PhD. Division of Pharmacodynamics, Keio University Faculty of Pharmacy, 1-5-30 Shibakoen, Minato-ku, Tokyo 105-8512, Japan. E-mail: taguchi-kz@pha.keio.ac.jp

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Competing interests

K. Matsumoto received grant support from Meiji Seika Pharma Co., Ltd. and Sumitomo Pharma Co., Ltd., and payment for lectures from Meiji Seika Pharma Co., Ltd. The other authors have no conflicts of interest to declare.

Author contributions

Y.I., W.T., X.L., N.K., and T.M. contributed to *in vivo* PD experiment. Y.I., V.T.G.C., Y.E., K.T., K.M. contributed to the interpretation of results. Y.I. wrote the manuscript with input from all authors.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request. Some data may not be made available because of privacy or ethical restrictions.

Abstract

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Experimental Approach: Based on Checkerboard MIC measurements, MIC_i of aztreonam against carbapenemase-producing*Klebsiella pneumoniae* in the presence of nacubactam was simulated. *In vivo* PD effect was evaluated by the bacterial count of thigh-infected mice after administered a combination of nacubactam and aztreonam. The mean change in the bacterial count obtained by*in vivo* PD study was plotted based on $\%f$ T>MIC_i and analysed using the Inhibitory Effect Sigmoid I_{max} Model.

Key Results: f T>MIC_icalculated from the PK experiments showed a high correlation with the bactericidal effect obtained in the PD experiments, suggesting that$f$ T>MIC_i is the optimal PK/PD parameter for aztreonam/nacubactam. The target values of$f$ T>MIC_i achieving growth inhibition, 1 $\log_{10}$-kill and 2 $\log_{10}$-kill, were 22, 38 and 75%, respectively.

Conclusion and Implications: The PK/PD analysis method proposed in this study is promising for determining practical PK/PD parameters in a combination antimicrobial therapy. In addition, this is the first report of aztreonam/nacubactam showing a potent *in vivo*therapeutic effect against carbapenemase-producing *K. pneumoniae* , particularly NDM-producing *K. pneumoniae* .

Bullet point summary

What is already known

β-lactams and β-lactamase inhibitors exert antibacterial activity in an interdependent manner.

PK/PD analysis method for interdependent β-lactam/β-lactamase inhibitor has not yet been established.

What this study adds

Instantaneous MIC (MIC_i) accurately represented β-lactamase inhibitor concentration-dependent changes in β-lactam susceptibility.

The new analytical approach,f T>MIC_i, and their target values were more practical and accurate.

Clinical significance

MIC_i reflects enzymatic type, inhibitory activity, antibacterial activity, and antibiotic additive and synergistic effects.

f T>MIC_i might theoretically be adaptable in antibiotic/antibiotic combination therapies as well as β-lactam/β-lactamase inhibitor.

INTRODUCTION

Since the late 1900s, the development of many antimicrobial agents has greatly improved human health and welfare. On the other hand, the increase in drug-resistant bacteria due to the inappropriate use of antimicrobials has become a problem worldwide. Recently, the Antimicrobial Resistance Collaborators reported that in 2019, 4.95 million deaths were associated with drug-resistant bacteria, of which 1.27 million were directly attributable to drug-resistant bacteria, strongly indicating that overcoming drug resistance is an important global healthcare challenge (Antimicrobial Resistance Collaborators, 2022). The O'Neill Report on Drug Resistance estimates that 10 million people will die annually from drug-resistant bacterial infections in 2050 if drug resistance increases at its current rate (O'Neill, 2016),(O'Neill, 2014). In particular, Gram-negative bacteria that have acquired resistance by producing β-lactamases that hydrolyse β-lactam antibiotics are positioned as a serious threat (Jacoby & Munoz-price, 2005). To treat patients with β-lactamase-producing gram-negative bacterial infections, the combination therapy of β-lactams and β-lactamase inhibitors, which inhibit the enzymatic inactivation of β-lactams by β-lactamases, has been the cornerstone of treatment of β-lactamase-producing Gram-negative bacterial infections in modern medical care.

In the clinical use of antimicrobials, pharmacokinetic/pharmacodynamic (PK/PD) parameters calculated using mouse infection models have contributed to determining evidence-based clinical doses of antimicrobials (Craig, 1998),(Andes & Craig, 2002),(Ambrose et al., 2006). In general, it is desirable to set the PK/PD parameters using specific minimum inhibitory concentrations (MICs) and pharmacokinetic parameters that can directly reflect the antimicrobial drug dose to be generalised to a wide variety of bacteria and doses. The PK/PD parameters for most antimicrobial monotherapies in clinical use are based on percentage of free time above MIC (%f T>MIC) or free area under the plasma concentration-time curve (f AUC)/MIC and free maximum concentration (f C_{max})/MIC to set the clinical dose (Ambrose et al., 2006). In contrast, the PK/PD parameters proposed for β-lactam/β-lactamase inhibitors are limited to$f$ T>C_T (Coleman et al., 2014). The C_T value, meaning 'the lowest concentration of β-lactamase inhibitor required to inhibit β-lactamase when used with a given dose of β-lactam' (Crass & Pai, 2019), is based on the fixed values of factors such as MIC of the bacterial strain, β-lactam administration method, β-lactamase genotype, gene expression levels. This makes it impossible to analyse drug efficacy with flexibility for bacteria with different MICs and concentrations of both drugs using$f$ T>C_T, thus preventing comprehensive clinical efficacy prediction. It is difficult to determine the PK/PD parameter that reflects the instantaneous variation of MICs in the presence of fluctuating blood concentrations of β-lactams/β-lactamase inhibitors over time, given the interdependency in the β-lactams/β-lactamase inhibitors drug efficacy. Therefore, PK/PD parameters for β-lactam/β-lactamase inhibitors using$f$ T>C_T values are not practical. Hence, the establishment of new PK/PD parameters incorporating the concept of MIC is desired.

We considered that using the instantaneous MIC (MIC_i) for PK/PD analysis of β-lactams/β-lactamase inhibitors could overcome this challenge. MIC_i is a mathematical modelling & simulation assessment concept that depends on β-lactamase inhibitor concentration and the sensitivity for β-lactam drugs that varies over time. This concept was first proposed by Bhagunde *et al.*(Bhagunde et al., 2012) in the imipenem/relebactam

*in vitro*Hollow-Fiber Infection Model. The utility of MIC_i has since been demonstrated for several β-lactam/β-lactamase inhibitor combinations (Wu et al., 2018),(Abodakpi et al., 2019 a),(Abodakpi et al., 2019 b),(Tam et al., 2021). Therefore, applying the MIC_i concept to PK/PD analysis in a murine infection model allows for flexible clinical dosing design, taking into account the interdependence of β-lactam/β-lactamase inhibitors. However, no reports have demonstrated the usefulness of PK/PD analysis using MIC_i.

Recently, nacubactam (OP0595), which is a DBO-type new β-lactamase inhibitor and has antibacterial activity, is being developed as a single drug to be co-administered with cefepime or aztreonam. This study aims to establish a PK/PD analysis method for β-lactam/β-lactamase inhibitors using MIC_i and investigate their superiority over$f$ $T>C_T$. For this purpose, we used nacubactam, a novel β-lactamase inhibitor currently under development, and aztreonam, a β-lactam drug. Furthermore, we used the PK/PD analysis method established in this study to explore the practical PK/PD parameters, their target values and optimal clinical doses of aztreonam/nacubactam against β-lactamase-producing Gram-negative bacteria.

MATERIALS and METHODS

Antimicrobial agents

Nacubactam hydrate was provided by Meiji Seika Pharma Co., Ltd. (Tokyo, Japan). Aztreonam powder (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) and aztreonam 1.0 g vials (Eisai Co., Ltd., Tokyo, Japan) were used for *in vitro* study, including a standard for concentration analysis and *in vivo* study, respectively.

Bacterial strains

Three strains of β-lactamase-producing *Klebsiella pneumoniae*listed in Table 1 were used in this study. *K. pneumoniae* ATCC BAA-2473 was obtained from American Type Culture Collection. Clinical isolates (*K. pneumoniae* MSC 21664 and MSC 21444) were provided by Meiji Seika Pharma Co., Ltd.

Checkerboard MIC and Effect Model analysis

According to Clinical and Laboratory Standards Institute guidelines (Clinical and Laboratory Standards Institute, 2018),(Clinical and Laboratory Standards Institute, 2021), checkerboard MICs of aztreonam/nacubactam were determined by broth microdilution method using cation-adjusted Mueller-Hinton broth (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). A 2-fold step dilution series of each drug was added to the wells of a 96-well plate at a volume of 1:1. Bacteria were added to the plate to a final concentration being approximately 2-8$\times10^5$ CFU mL^{-1}. After incubating at 37 °C for 20 h, the lowest concentration of aztreonam with each nacubactam concentration, at which no bacterial growth was observed, was defined as MIC.

The results obtained by checkerboard assay for each strain were plotted and analysed using the Inhibitory Effect Sigmoid$I_{\max}$ Model [equation (1)] to draw an approximate curve representing the dose-response relationship of the aztreonam MIC to nacubactam concentration.

$$\log_2(\mathrm{MIC}) = \log_2(\mathrm{MIC}_0) - (I_{\max} \times {C_{NAC}}^u) \times ({C_{NAC}}^u + {IC_{50}}^u)^{-1} \quad \text{Eq.(1)}$$

where MIC_0 is the MIC of aztreonam in the absence of nacubactam, C_{NAC} is the concentration of nacubactam (mg L^{-1}), $I_{\max}$ is the maximum inhibitory effect, IC_{50} is the concentration of nacubactam at which 50% of the maximum inhibition, u is the sigmoid coefficient.

Animals

Male ICR mice (Sankyo Labo Service Corporation, Inc., Shizuoka, Japan) were used for all animal experiments. The mice were acclimated for 1 week under a 12-hour light/dark cycle with feeding and watering *ad libitum* and used for the experiment at 5 weeks of age. The mice were intraperitoneally injected with cyclophosphamide (Shionogi & Co., Ltd., Osaka, Japan) for 4 days (150 mg kg^{-1}) and the day (100 mg kg^{-1}) before the experiment to decrease their neutrophils. Protocols of all animal experiments were approved by the Institutional Animal Care and Use Committee of Keio University (No. 19037) and the Animal Experiment Management Committee, Pharmaceutical Research Center, Meiji Seika Pharma Co., Ltd.

PK analysis

Aztreonam/nacubactam was subcutaneously administered to neutropenic mice. At 5, 15, 30, 60, 120, 240, and 300 min after administration, blood samples were collected through cardiac puncture under anesthesia (n = 3 per time point). Immediately after blood sampling, the mice were euthanised by cervical dislocation. The blood samples were centrifuged to separate the plasma and stored at -80 °C until analysis. PK parameters were calculated by compartmental model analysis of free plasma nacubactam and aztreonam concentrations using Phoenix WinNonlin^{TM} (ver. 8.0, Certara, NJ, USA).

High-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis

The concentration of antibiotics in each plasma sample was measured using a validated LC-MS/MS method. Detailed measurement conditions are summarised in Table S1. The protein binding rates of nacubactam and aztreonam in mouse plasma were determined by the ultrafiltration method. In short, plasma containing nacubactam and aztreonam at concentrations of 1, 10, and 400 mg L^{-1} (n = 3 per concentration) was incubated at 37 °C for 30 min and then set into Centrifree® 4104 Ultrafiltration Centrifugal Filters (Millipore, Bedford, MA, USA). The filtrate was obtained by centrifugation at 1,500 g for 5 min. The concentrations of the antibiotics in the initial sample and the filtrate were measured using LC-MS/MS, and the protein binding rate was calculated.

PD study

Neutropenic mice were infected intramuscularly with 100 μL of inoculum bacteria suspension at a concentration of 1×10^6 to 10^7 CFU mL^{-1} in the left thigh 2 hrs before drug administration. A combination of nacubactam (0, 1.2, 3.6, 12, 36, 120, 360, 1,200 mg kg^{-1} day^{-1}) and aztreonam (0, 1,200, 2,400, 4,800 mg kg^{-1} day^{-1}) were subcutaneously administered from 2 to 26 hrs after bacterial inoculation (n = 3 per dose). In the dose fractionation study, aztreonam was administered every 2 hrs (q2h). In contrast, nacubactam was administered at a variable interval schedule (q2h, q4h, and q8h) from 2 to 26 hrs after bacterial inoculation (Fig. S1). In a dose-ranging study, each drug was administered in 12 divided doses (q2h) from 2 to 26 hrs after bacterial inoculation (Fig. S1). The control mice were euthanised by cervical dislocation at 0 h (2 h after bacterial inoculation). Other neutropenic mice were euthanised 24 h after initial drug dosing (26 h after bacterial inoculation). Then, the left thigh was aseptically collected and homogenised (1,800 rpm, 120 s, 18 °C) using Multi-Beads Shocker (Yasui Kikai Corp., Osaka, Japan). Serial dilution series of each homogenate were prepared, and an aliquot of each suspension was applied to Mueller-Hinton agar plates. After incubation at 37 °C for about 20 hrs, the number of colonies that grew was measured. The lowest detection limit in this method was 2.20 $\log_{10}$ CFU $thigh^{-1}$.

PK/PD analysis with$f$T>MIC$_i$

The time-course aztreonam MIC_i after nacubactam administration was calculated by applying the time-course free plasma nacubactam concentration to the dose-response relationship equation. The percentage of time that the free plasma aztreonam concentration exceeded the MIC_i was defined as $\%f$ T>MIC_i (Fig. 1). The mean change in the bacterial count of each group obtained by *in vivo* PD study was plotted based on $\%f$ T>MIC_i and analysed using the Inhibitory Effect Sigmoid I_{max}Model [equation (2)].

$$E = E_0 - I_{max} \times (f\ \mathrm{T}>\mathrm{MIC_i})^u \times \{(f\ \mathrm{T}>\mathrm{MIC_i})^u + \mathrm{IC_{50}}^u\}^{-1} \quad \text{Eq.(2)}$$

where E is the changes in the bacterial count, E_0 is bacterial counts at f T>MIC_i = 0%, I_{max} is the maximum inhibitory effect, IC_{50} is the f T>MIC_i at which 50% of the maximum inhibition and u is the sigmoid coefficient.

PK/PD analysis with fT>C$_T$

The percentage of time that the free plasma nacubactam concentration at each dose exceeded the C_T value (0.125, 0.25, 0.5, 1, 2, and 2.5 mg L^{-1}) was calculated as $\%f$ T>C_T. The mean change in the bacterial count of each group obtained by *in vivo* PD study was plotted based on $\%$ f T>C_T and analysed by the Inhibitory Effect Sigmoid I_{max}Model [equation (2)].

Simulation in clinical dosing

Using PK data of nacubactam (Mallalieu et al., 2020) and aztreonam (Scully et al., 1983),(Kita et al., 1986) in healthy subjects, $\%f$ T>MIC_i and $\%f$ T>C_T against strains used in this study were calculated in the case that aztreonam (0.5 g, 1 g, 2 g) and nacubactam (0.05 g, 0.15 g, 0.5 g, 1 g, 2 g) were co-administered every 8 hrs. In the simulation case of $\%f$ T>C_T, aztreonam 1,200, 2,400, and 4,800 mg kg^{-1} day^{-1} in mice were calculated as aztreonam 0.5 g q8h, 1 g q8h, and 2 g q8h in human, respectively, because of similar kinetic transition.

RESULTS

Checkerboard MIC and Effect Model analysis

The β-lactamase genotype of each bacterial strain and the MIC values of aztreonam/nacubactam alone and nacubactam MIC in the presence of 4 mg L^{-1} of aztreonam for each bacterial strain are shown in Table 1. Fig. 2 shows the dose-response relationship of aztreonam MIC to nacubactam concentration for each strain analysed by the Inhibitory Effect Sigmoid I_{max} Model. All strains were resistant to aztreonam alone, but the combination of aztreonam and nacubactam decreased MIC in a nacubactam concentration-dependent manner. Interestingly, nacubactam alone also showed slight antibacterial activity against all bacterial strains.

PK analysis of antimicrobial agents

The PK profiles of nacubactam and aztreonam in neutropenic mice are shown in Fig. 3. The plasma concentration curve for nacubactam and aztreonam best fitted the two-compartment model. The PK parameters of each drug analysed from the plasma concentration curve are listed in Table S2. C_{max} and AUC of each drug showed good linearity within the dose range tested (Fig. S2). The plasma protein binding rates of nacubactam and aztreonam were similar among the three concentrations (Table S3), with averages of 3.87% and 57.1% for nacubactam and aztreonam, respectively. The average values were used for PK/PD analysis.

PD study

An *in vivo* dose fractionation study in neutropenic mice infected with *K. pneumoniae* ATCC BAA-2473 thighs showed that aztreonam and nacubactam alone hardly reduced viable bacterial counts. However, the aztreonam/nacubactam combination showed a nacubactam dose-dependent bacterial count reduction and a strong bactericidal effect of up to 2 $\log_{10}$ CFU $thigh^{-1}$ compared to the control group (Fig. 4).

The *in vivo* dose-ranging study of aztreonam/nacubactam was assessed in neutropenic thigh-infected mice with *K. pneumoniae*MSC 21664 and *K. pneumoniae* MSC 21444, administering aztreonam/nacubactam at various doses every 2 hrs (Fig. 5). In both strains, nacubactam and aztreonam alone showed a slight reduction in bacterial counts, but the combination of both antibiotics provided a potent bactericidal effect. The maximum bactericidal effect in neutropenic mice infected with *K. pneumoniae* MSC 21664 and*K. pneumoniae* MSC 21444 was about 3 $\log_{10}$ and 1 $\log_{10}$ CFU $thigh^{-1}$, respectively.

PK/PD analysis with$f$T>MIC_i

The values of f T>MIC_i in each dose group calculated from the PK data were plotted against the change in bacterial counts obtained in the PD study (Fig. 6). The results showed a high correlation (R^2=0.868) between the change in viable bacterial counts and$f$ T>MIC_i following aztreonam/nacubactam administration. The growth inhibition and bactericidal effect target values were further analysed using the Inhibitory Effect Sigmoid I_{max} Model. The target values of f T>MIC_i required to achieve growth inhibition, 1 $\log_{10}$-kill and 2 $\log_{10}$-kill, were 22%, 38%, and 75%, respectively.

PK/PD analysis with fT>C_T

The values of f T>C_T in each dose group calculated from the PK data were plotted against the change in bacterial counts obtained in the PD study. TheR^2 value in each C_T of nacubactam was analysed using the Inhibitory Effect SigmoidI_{max} Model (Fig. S3-5). Fig. 7 summarised the relationship between C_T of nacubactam andR^2 values. The C_T values with the highest R^2 value for *K. pneumoniae*ATCC BAA-2473

when combined with aztreonam 1,200, 2,400 and 4,800 mg kg^{-1} day^{-1} were 2, 1 and 0.125 mg L^{-1}, respectively (Fig. 7A). For *K. pneumoniae*MSC 21664, the C_T value with the highestR^2 values was 0.125 mg $L^{-1}$for all doses (Fig. 7B). For *K. pneumoniae* MSC 21444, C_T = 0.25 mg L^{-1} with aztreonam 1,200 mg kg^{-1} day^{-1} and C_T= 0.125 mg L^{-1} with 2,400 and 4,800 mg kg^{-1} day^{-1} had the highestR^2 values (Fig. 7C).

Simulation in clinical dosing

Using PK data from healthy adults,f T>MIC_i (%) for aztreonam (0.5 g, 1 g, 2 g) and nacubactam (0.05 g, 0.15 g, 0.5 g, 1 g, 2 g) given in combination every 8 hrs is shown in Table 2. For NDM-1 β-lactamase producing *K. pneumoniae* ATCC BAA-2473, growth inhibition was achieved at the lowest dose combination (aztreonam 0.5 g q8h and nacubactam 0.05 g q8h), and bactericidal effects were achieved at all other doses. In particular, 2 log_{10}-kill was achieved at doses above nacubactam 0.5 g q8h regardless of aztreonam dosage. A 2 log_{10}-kill was achieved against IMP-6 β-lactamase producing *K. pneumoniae* MSC 21664 at all simulated doses. For OXA-48 β-lactamase producing *K. pneumoniae* MSC 21444, bactericidal efficacy was achieved at all doses and 2 log_{10}-kill at doses of nacubactam 0.5 g q8h and above. In all cases, f T>MIC_i = 100% was reached at doses of nacubactam 1 g q8h and above, suggesting that the combination of aztreonam/nacubactam provides adequate therapeutic efficacy against β-lactamase-producing *K. pneumoniae* infection in human.

The f T>C_T, when aztreonam (0.5 g, 1 g, and 2 g) and nacubactam (0.05 g, 0.15 g, 0.5 g, 1 g, and 2 g) are co-administered every 8 hrs, is also calculated and summarised in Table 3. The C_T value with the highestR^2 value at each dose was used in the calculations (Fig. 7). For NDM-1-positive *K. pneumoniae* ATCC BAA-2473, the minimum combination of aztreonam 0.5 g q8h and nacubactam 0.05 g q8h achieved growth inhibition, and all other doses achieved bactericidal effects.

For IMP-6-positive *K. pneumoniae* MSC 21664, 2 log_{10}-kill was achieved at all simulated doses as well as f T>MIC_i. As for OXA-48-positive*K. pneumoniae* MSC 21444, all nacubactam dosages achieved growth inhibition when co-administered with aztreonam 0.5 g q8h and 1 log_{10}-kill when co-administered with aztreonam 1 g or 2 g q8h. Interestingly, the bactericidal effect simulated by$f$ T>C_T against NDM-1-positive*K. pneumoniae* ATCC BAA-2473 and OXA-48-positive *K. pneumoniae* MSC 21444 was estimated lower than$f$ T>MIC_i.

DISCUSSION

The PK/PD parameters of β-lactam/β-lactamase inhibitor combos are challenging to determine. This is because some β-lactamase inhibitors have antibacterial activity in addition to protecting β-lactams from β-lactamases, and the antimicrobial effects of various combinations are interdependent. There is a need to establish accurate analytical methods to determine the PK/PD parameters of β-lactam/β-lactamase inhibitor combinations because inappropriate analytical methods or models with low predictive accuracy risks creating a dissociation between susceptibility data *in vitro* and actual clinical efficacy. The current research shows how the MIC_i concept may be used in conventional PK/PD analysis to produce PK/PD parameters that are more practical, versatile, and accurate than those produced using$f$ T>C_T.

The first generation β-lactamase inhibitor combination piperacillin/tazobactam has been tested for the MIC of piperacillin in a fixed concentration of tazobactam of 4 mg L^{-1}, at a dose ratio of 8:1. On the other hand, amoxicillin/clavulanate is tested for susceptibility at a fixed 2:1 ratio, although several formulations with ratios of 2:1, 4:1 and 7:1 are used and may not correlate with efficacy. This *in vitro* and *in vivo* difference in combination ratios may be due to the fluctuating susceptibility of β-lactam drugs in response to changing inhibitor concentrations over time is ignored during *in vivo* analysis. Optimisation of$f$ T>C_T is based on the assumption that the susceptibility of β-lactam drugs is constant in the presence of β-lactamase inhibitors. However, MICs for clinical strains are not incorporated into the dosing design and do not adequately reflect changes in β-lactam drug susceptibility caused by different β-lactamase inhibitor concentrations.

In the present study, the MIC curve for aztreonam varied significantly in the therapeutic concentration range of nacubactam (approximately 0.1-10 mg L^{-1}) (Fig. 2). It confirmed the importance of the PK/PD parameters for β-lactam drugs/β-lactamase inhibitors should consider changes in susceptibility under different

β-lactamase inhibitor concentration conditions. On the other hand,f T>MIC_i can reflect changes in β-lactam susceptibility and changes in β-lactamase inhibitor concentration. In order to compare the accuracy of these two PK/PD parameters, an attempt was made to predict clinical efficacy in humans using each analysis method. The results showed that for *K. pneumoniae* ATCC BAA-2473 and *K. pneumoniae* MSC 21444, the bactericidal effect simulated by$f$ T>C_T tended to be estimated lower than f T>MIC_i (orange area of 2 $\log_{10}$-kill in Tables 2 and 3). This may be due to underestimating the PK/PD predictions based on$f$ T>C_T. Higher aztreonam concentrations are clinically effective even when nacubactam concentrations are below the C_T value. Therefore,f T>MIC_i is considered a more accurate PK/PD parameter than f T>C_T. However, the f T>MIC_i approach requires time-consuming susceptibility testing andE_{max} modelling analysis.

It is worth noting that analyses based on$f$ T>MIC_i have practical versatility because they do not need to consider all factors. The MIC curve reflects enzyme type, inhibitory activity, antimicrobial activity, and the additive and synergistic effects of antimicrobial agents. Indeed, the analysis using f T>MIC_i showed a good correlation (R^2=0.868) for three β-lactamase enzyme-producing strains of different genotypes, confirming the *in vivo* validity of the f T>MIC_i-based analysis. On the other hand, a similar analysis using$f$ T>C_T confirmed that the C_T values of nacubactam depended on the strain or the dose of aztreonam used in combination (Fig. 7). These results suggest that PK/PD analysis using f T>$C_T$lacks practical versatility in determining a single target value for various clinical isolates.

The novel DBO-based β-lactamase inhibitor, nacubactam, is a potent inhibitor of Ambler class A (e.g. KPC and ESBL) and C (AmpC) β-lactamases but weakly inhibits class D (OXA) β-lactamases (Morinaka et al., 2015),(Morinaka et al., 2016). In addition, nacubactam exhibits antibacterial activity through binding to the bacterial penicillin-binding protein (PBP)-2. Furthermore, unlike first-generation β-lactamase inhibitors (e.g. sulbactam, clavulanic acid, tazobactam), second-generation DBO-based β-lactamase inhibitors, including nacubactam, do not have a β-lactam structure and are not subject to β-lactamase degradation. Therefore, they are expected to be a new treatment option for carbapenemase (Metallo-β-lactamase)-producing bacteria, including Class B (NDM and IMP) (Livermore et al., 2015),(Mushtaq et al., 2018), because no other established treatment is available. In the current checkerboard MIC study, aztreonam/nacubactam showed good combination efficacy against Class B and Class D β-lactamase-producing bacteria such as NDM-1, IMP-6, and OXA-48 β-lactamase producing *K. pneumoniae* (Table 1). Furthermore, the MIC of nacubactam against all bacterial species in the presence of 4 mg L^{-1} aztreonam, which reflects the minimum nacubactam concentration to achieve the microbiological breakpoint (Clinical and Laboratory Standards Institute, 2021), can be easily achieved in clinical practice, indicating aztreonam/nacubactam *in vivo* potent antimicrobial effect is obtainable. Indeed, the following two lines of evidence in this study support the potent β-lactamase inhibition and antibacterial activity of nacubactam against β-lactamase-producing*K. pneumoniae* . Firstly, aztreonam/nacubactam combination therapy was effective in a mouse model against NDM-1 and IMP-6, OXA-48-producing*K. pneumoniae* in a nacubactam dose-dependent manner, despite aztreonam monotherapy being ineffective. Secondly, the dose fractionation study showed a concentration-dependent bactericidal effect of nacubactam irrespective of the number of doses administered. Thus, the present study provides the first evidence of a satisfactory therapeutic effect of nacubactam against refractory β-lactamase-producing *K. pneumoniae* , particularly the *in vivo* antibacterial effect of nacubactam against NDM-producing bacteria.

There are two drawbacks to this research. The first is that all PD trials only measured activity against fixed inoculum levels (approximately 10^6 CFU mL^{-1}). The effect of greater inoculum levels on aztreonam/nacubactam drug efficacy is unknown. Due to the inoculum effect, several antimicrobials have lower antimicrobial effectiveness at higher initial inoculum levels (Brook, 1989). Some β-lactam/β-lactamase inhibitor combinations, on the other hand, have been less influenced by the inoculum effect. Ceftazidime/tazobactam, for example, showed decreased action at high bacterial levels, but ceftazidime/avibactam was said to be unaffected by inoculum levels (Tam et al., 2021). The causes for this are unknown. However, the inoculum effect could be related to avibactam's reversible inhibitory action and resistance to enzymatic hydrolysis. Because nacubactam inhibits in the same way that avibactam does, it may be less vulnerable to the inoculum effect. On this point, more evidence is required. Finally, clinical target values were predicted using PK data from healthy adults. Although it would be ideal for simulating more clinically relevant settings using PK

data from patients with infections, PK data for nacubactam in patients with infections is not yet publically available. Nacubactam PK data in patients with infections are awaited.

CONCLUSIONS

Finally, adopting MIC_i, a comprehensive concept, a new PK/PD analysis approach for β-lactam/β-lactamase inhibitor combination therapy was developed. In comparison to existing PK/PD parameters (f $T{>}C_T$), the PK/PD parameters (f $T{>}MIC_i$) and their target values in this newly designed PK/PD analysis approach were more practical, generic, and accurate. PK/PD analysis employing$f$ $T{>}MIC_i$ might theoretically be used in β-lactam/β-lactamase inhibitor combination therapy and antimicrobial/antimicrobial combination therapy. Furthermore, this is the first report of aztreonam/nacubactam being effective against carbapenemase-producing *K. pneumoniae* , particularly NDM-producing*K. pneumoniae* . By improving the treatment of drug-resistant bacterial infections, this study's findings should help improve human health and welfare.

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