***Title*:**

*In vitro* effectiveness of biapenem against IMP-producing Enterobacteriaceae

***Running Title*:**

Biapenem for IMP-producing CRE

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***Abstract***

The options available for treating infections with carbapenemase-producing Enterobacteriaceae (CPE) are limited; with the increasing threat of these infections, new treatments are urgently needed. Biapenem (BIPM) is a carbapenem, and limited data confirming its *in vitro* killing effect against CPE are available. In this study, we examined the minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) of BIPM for 14 IMP-1–producing Enterobacteriaceae strains isolated from the Okayama region in Japan. The MICs against almost all the isolates were lower than 0.5 μg/mL, indicating susceptibility to BIPM, while about half of the isolates were confirmed to be bacteriostatic to BIPM. However, initial killing to the 99.9% reduction were observed in 7 out of 8 strains as a result of time-killing assay. Despite the small data set, we concluded that the *in vitro* efficacy of BIPM suggests that the drug could be a new therapeutic option against infection with IMP-producing CPE.

## *Introduction*

The emergence and global spread of antimicrobial resistance, especially that of carbapenem-producing Enterobacteriaceae (CPE), is a major public health concern.[1,2] Carbapenemases are diverse in nature and comprise the *bla*$_{NDM}$, *bla*$_{KPC}$, *bla*$_{IMP}$, *bla*$_{VIM}$, and *bla*$_{OXA-48}$ families,[2] which complicate diagnostic and therapeutic approaches. Among the various genetic types, strains producing metallo-beta-lactamases (MBL), such as *bla*$_{NDM}$, *bla*$_{IMP}$, and *bla*$_{VIM}$, show higher resistance to carbapenems, and only limited treatment options are available for infections with these strains.[3] However, because of the lack of research funding and the difficulty of searching for research seeds, hardly any new antibiotics have been developed in recent times. Therefore, in this era of antibiotic shortage, it is necessary to seek for alternative strategies to manage CPE infections, and an exploration for effective drugs among the existing antibiotics will be useful for developing new therapeutic strategies.

Presently, there are five carbapenem-class intravenous antimicrobials available for use in clinical settings in Japan: imipenem, meropenem (MEPM), doripenem, panipenem, and biapenem (BIPM). Among them, BIPM was developed in the 1990s as a pharmacokinetically stable agent with high resistance to hydrolysis by renal dehydropeptidase-I (DHP-I) and that has

*in vitro* antibacterial activity against gram-negative, gram-positive, and anaerobic bacteria at levels comparable to other carbapenems.[4] Later reports suggested that *in vivo* and *in vitro* efficacies of BIPM against *Pseudomonas aeruginosa* were possibly superior to those of MEPM and imipenem.[5,6] A biochemical study suggested that the low electrostatic polarity of the molecular residual group in BIPM helps drug permeation into bacterial cells via the outer membrane porin.[7] An enzymology study demonstrated a low kcat/Km ratio for MBLs, including IMP-1, VIM-2, and NDM-1 type β-lactamases, pertaining to BIPM.[8] Although BIPM seems a good therapeutic candidate for the management of CPE infections, data about its *in vitro* effectiveness data are lacking. In this study, we aimed to investigate the *in vitro* killing efficiency of BIPM against IMP-producing Enterobacteriaceae, which are predominant in Japan.[9-12]

## *Methods*

### *Bacterial strain*

As shown in **Table 1**, we exposed $bla_{\text{IMP-1}}$-harboring Enterobacteriaceae isolated from Okayama University Hospital (1 *Klebsiella pneumoniae*, 2 *Klebsiella oxytoca*, and 5 *Enterobacter cloacae* complex) and Tsuyama Chuo Hospital (7 *Klebsiella pneumoniae* and 1 *Enterobacter kobei*) in Okayama, Japan, to BIPM.

78

79 ***Minimum inhibitory concentrations (MIC) and Minimum bactericidal concentrations (MBC)***

80 MICs of MEPM and BIPM were determined by a broth microdilution method using
81 round-bottomed 96-well microtiter plates based on the Clinical and Laboratory Standards Institute
82 (CLSI) document M07-A10 (Dry Plate Eiken, Eiken Chemical Co., Ltd.). The MIC was defined
83 as the lowest concentration of an antimicrobial agent that inhibits bacterial growth after 18 hours
84 incubation. The MBCs of BIPM were further determined by subculturing broth dilutions at or
85 above the MIC onto Muller-Hinton agar plates. The MBC was defined as the lowest broth dilution
86 of the antimicrobial that formed no colony on the plate. A bactericidal effect was defined as the
87 MBC being no more than four times the MIC. When the MBC was more than that, it was
88 considered a bacteriostatic effect.

89

90 ***Time-kill assay***

91 To further confirm the antimicrobial activity of BIPM, a time-killing assay was
92 performed using eight isolates from OUH. Test tubes with a volume of 10 mL were used for
93 growth control and individual antimicrobials (1 μg/mL each of MEPM and BIPM) in a final
94 volume of 4 mL. The initial inoculum was set at 1-5 × $10^5$ colony-forming unit (CFU)/mL and

95 performed incubation using the BBL™ Muller-Hinton II broth (Becton, Dickinson and Company,
96 Sparks, MD, USA). After shaking culture at 130 rpm and 37°C in ambient air, aliquots were
97 obtained from each tube and inoculated on Muller-Hinton agar plates by plating serial 10-fold
98 dilutions at 2, 4, 6, 8, and 24 hours after drug supplementation. The plates were incubated
99 overnight in ambient air at 37°C, and the number of viable colonies was counted. The lower
100 limitation for the assay was 20 CFU/mL. Efficacies were evaluated whether or not the viable
101 bacterial count reduced below 99.9% compared to the initial bacterial count at each examined
102 point. Every time-killing test was performed in triplicate.
103
104 ***Results***
105 The MIC values for MEPM among the 14 strains of IMP-1–producing
106 Enterobacteriaceae ranged from 2 to >16 μg/mL. For 13 out of the 14 strains (92.9%), the MIC
107 of BIPM was ≤0.5 μg/mL, indicating susceptibility, based upon the information in the guideline
108 established by the Japanese Society of Chemotherapy[13] (**Table 1**). Cumulative curves show an
109 apparent difference in the MIC distribution between MEPM and BIPM (**Fig. 1**). No isolate was
110 susceptible to MEPM according to the CLSI breakpoint values. Of 14 isolates, 6 strains (42.9%)
111 showed the bacteriostatic effect; those of 8 isolates were undetermined due to a limitation of MIC

measurement range.

The time-killing assay was then performed to evaluate whether BIPM actually exhibited practical efficiency against these bacteriostatic strains. Eight isolates from OUH including 4 bacteriostatic strains to BIPM revealed that 1.0 μg/mL BIPM showed 99.9% reduction within first 6 hours in seven isolates, excepting strain OUH016 (**Fig. 2**). Among the seven strains with the rapid killing, six regrew after 24 hours, while strain OUH023 was completely eradicated. On the other hand, 1.0 μg/mL MEPM did not achieve the 99.9% reduction in any of the isolates.

## Discussion

In this study, we demonstrated that the distribution of MICs of BIPM was lower than those of MEPM in the bacteria population among IMP-producing Enterobacteriaceae, suggesting the potential effectiveness of BIPM. The time-killing assay corroborated the *in vitro* superiority of BIPM over MEPM, although bactericidal efficacy may not be expected. In this era of antimicrobial resistance, recycling of approved antimicrobials can be a solution for the development of new treatment strategies. Our study provides one such possibility.

The potential utility of BIPM against CPE has been suggested in the past decade, although not extensively. Livermore *et al.* reported that compared to the MICs of MEPM, imipenem, and doripenem, BIPM had lower MIC levels for NDM-, VIM-, and IMP-producing Enterobacteriaceae, mainly isolated from the United Kingdom.[8] In addition, BIPM, as a substrate for IMP-1, VIM-2, and NDM-1 MBL, has a lower hydrolysis rate than do the other three carbapenems, which was explained by its comparably lower $k_{cat}/K_m$ values.[8] Further, BIPM administration at a clinically available concentration (3.0 μg/mL) was confirmed to be effective against these isolates when combined with ME1071, a suicide inhibitor.[8] A pharmacokinetics study showed that administration of 300 mg of BIPM every 8 hours can result in a serum concentration at 24.7 ± 4.97 μg/mL.[14] This value was practically compatible with the use of 1.0 μg/mL BIPM in this study. On the basis of previously published data and data from our current study, we believe that BIPM can be applied for clinical use in treating CPE infections.

This study has several limitations. First, the small sample size and regionally localized subjects for the study may limit the generalization of the results. Second, we solely focused on IMP-producing organisms, and the *in vitro* effects of BIPM against other types of MBLs were not evaluated. For clinical use, further evaluation of the antimicrobial potential of BIPM in *in vivo*

settings is necessary.

## Conclusion

In summary, BIPM, a forgotten carbapenem class antimicrobial, showed *in vitro* effectiveness against IMP-type CPE. We suggest that BIPM has potential as a new therapeutic option for managing infections with IMP-1–producing CPE and could be a candidate for a recycle use of existing antimicrobials to combat against emerging antimicrobial resistant pathogens.

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## Author Contribution

All authors meet the ICMJE authorship criteria: K. Gotoh and H. Hagiya were the principal investigators; The contributors O. Matsushita, F. Otsuka were in charge of the organizations and coordination of the study; H. Hagiya created the study design; K. Gotoh M. Miyoshi, B. Mayura, and K. Iio carried out the measurements; K. Gotoh and H. Hagiya were in charge of data analysis; K. Gotoh drafted the manuscript and all authors contributed to writing the final manuscript.

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