

**© Free Author
Copy - for per-
sonal use only**PLEASE NOTE THAT ANY
DISTRIBUTION OF THIS AR-
TICLE WITHOUT WRITTEN
CONSENT FROM S. KARGER
AG, BASEL IS A VIOLATION
OF THE COPYRIGHT.Upon request a written per-
mission to distribute the PDF
file will be granted against
payment of a permission fee
depending on the number of
accesses required. Please
contact Karger Publishers,
Basel, Switzerland at
permission@karger.ch

Postantibiotic and Post-Beta-Lactamase Inhibitor Effect of Carbapenems Combined with EDTA against *Pseudomonas aeruginosa* Strains Producing VIM-Metallo Beta-Lactamases

B. Bedenić^a J. Vraneš^b M. Sviben^c N. Beader^a S. Kalenić^a^aDepartments of Microbiology, School of Medicine, University of Zagreb and Clinical and Molecular Microbiology, Clinical Hospital Center Zagreb, ^bDepartment of Microbiology, Zagreb Institute of Public Health, and School of Medicine, University of Zagreb, ^cDepartment of Parasitology, Croatian National Institute of Public Health, Zagreb, Croatia

Key Words

Postantibiotic effect · Post-beta-lactamase inhibitor effect · Meropenem · Imipenem · EDTA

Abstract

Background and Aim: Postantibiotic effect (PAE) is a delay of bacterial growth after short exposure to antibiotics. The phenomenon of continuing suppression of bacterial growth after removal of β -lactamase inhibitors is termed post- β -lactamase inhibitor effect (PLIE). Recently, *Pseudomonas aeruginosa* strains producing metallo- β -lactamases were described in many countries of the world. The aim of the study was to investigate the PLIE of carbapenems in combinations with EDTA against VIM-MBL-positive strains of *P. aeruginosa*. **Methods:** The experiments were performed on two *Pseudomonas aeruginosa* isolates, one producing VIM-1 and the other producing VIM-2 metallo- β -lactamase. Minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBC) of imipenem and meropenem alone and combined with EDTA, time-kill curves, PAE and PLIE were performed as described previously. **Results:** The duration of PAE with meropenem combined with EDTA at 8 \times MIC was longer against both VIM-1 and VIM-2 producer than that of imipenem with EDTA on VIM-1- and VIM-2-positive strains. The duration of PLIE was similar on both strains of *P. aeruginosa* regardless of the sort of carbapenem. At lower

concentrations, meropenem with EDTA induced slightly longer PAE and PLIE than imipenem with EDTA. **Conclusions:** This study has shown that EDTA combined with carbapenems produced a significant PLIE on VIM-MBL-positive *P. aeruginosa* strains. The results do not have any clinical relevance so far since metal chelators such as EDTA are not used as therapeutic agents due to their toxicity.

Copyright © 2008 S. Karger AG, Basel

Introduction

Bacterial resistance is a growing phenomenon and causes therapeutic difficulties in everyday practice [1]. The production of β -lactamases is still the main mechanism of resistance to β -lactams in Gram-negative bacteria [1]. Postantibiotic effect (PAE) is a delay of bacterial growth after short exposure to antibiotics [2]. The phenomenon of continuing suppression of bacterial growth after removal of β -lactamase inhibitors is termed post- β -lactamase inhibitor effect (PLIE) [3]. PAE and PLIE enable intermittent dosing of the antibiotics [4, 5]. *Pseudomonas aeruginosa*, a significant hospital pathogen, exhibits significant PAE with aminoglycosides and fluoroquinolones, but no or negative PAE with β -lactams except for carbapenems [6, 7]. The existence of PAE for carbapenems against *P. aeruginosa* has already been report-

ed [8, 9]. Recently, *P. aeruginosa* isolates producing metallo- β -lactamases (MBLs), enzymes capable of hydrolyzing carbapenems, have been reported with increasing frequency in many countries of the world [10, 11]. It was also found that administration of carbapenems select VIM and IMP β -lactamases [12]. However, there are no published reports on PAE of carbapenems combined with metal chelators against MBL-positive strains of *P. aeruginosa* which are resistant to carbapenems and all other β -lactams except for aztreonam. The addition of metal chelators such as EDTA, fenantroline and mercaptopropionic acid restores the susceptibility to carbapenems of MBL-positive strains of *P. aeruginosa* [13]. The aim of the study was to investigate the PLIE of carbapenems in combinations with EDTA against MBL-positive strains of *P. aeruginosa*. The PLIE was previously described for amoxicillin plus clavulanate against *Haemophilus influenzae*, *Moraxella catarrhalis* and *Klebsiella pneumoniae* [4, 5] and for sulbactam plus ceftazidime against ESBL-positive *K. pneumoniae* and *Escherichia coli* [3], but there are no reports on PLIE of metal chelators against MBL producers. Contrary to suicide inhibitors such as clavulanic acid or sulbactam, metal chelators such as EDTA are not applied as therapeutic agents due to their toxicity. However, it is useful to investigate if there are any post-exposure effects produced by these agents in order to clarify the pharmacodynamic mechanisms of metallo β -lactamase inhibition.

Materials and Methods

Strains

The VIM-1-positive strain of *P. aeruginosa* was kindly provided by Dr. A. Mazzariol (Department of Pathology, Section of Microbiology, University of Verona, Italy) and the VIM-2-positive strain was isolated at the Clinical Hospital Center Zagreb.

Susceptibility Testing

Minimum inhibitory concentrations (MICs) of imipenem and meropenem alone and combined with EDTA were determined by the broth microdilution method according to CLSI [14]. Twofold serial dilutions of the antibiotics were prepared in cation-supplemented Mueller-Hinton broth in microtiter plates. Minimum bactericidal concentration (MBC) was defined as the lowest antibiotic concentration that reduced the inoculum by 99.9%.

Time-Kill Curves

An overnight culture of test strains was diluted 1:10 in cation-supplemented Mueller-Hinton (MH) broth, incubated for 2 h in a shaking water bath to reach the logarithmic phase and then diluted in 20 ml of the same medium to yield an inoculum of approximately 10^7 CFU/ml. Antibiotics (carbapenem or meropenem plus EDTA) were added at a concentration of $8 \times \text{MIC}$ or $2 \times$

MIC. EDTA was added at a fixed concentration of 0.4 mM. Bacterial counts were determined at time 0, every hour for 8 h, and then at 24 h, using 10-fold dilutions in phosphate-buffered saline (PBS) [5].

Determination of PAE

An overnight culture of test strains was diluted 1:10 in cation-supplemented MH broth, incubated for 2 h in a shaking water bath to reach the logarithmic phase and then diluted in 20 ml of the same medium to yield approximately 10^7 CFU/ml. Carbapenem (imipenem or meropenem) in combination with EDTA was added at a concentration corresponding to $8 \times \text{MIC}$ or $2 \times \text{MIC}$ and incubated for 2 h in a shaking water bath. To remove the antibiotics, culture was centrifuged at 12,000 rpm, the pellet was washed twice with PBS, resuspended in the same volume of pre-warmed Mueller-Hinton broth and incubated for 8 h and then up to 24 h. An unexposed culture of the same strains was used as a control. Bacterial counts were determined at time 0, immediately before and after centrifugation and every hour for 8 h, and then at 24 h, using 10-fold dilutions in PBS. 100 μl of each dilution was seeded on Mueller-Hinton agar and the plates were incubated at 37°C in ambient air. The 100- μl sample was applied to the plate with a micropipette and spread over the whole surface of the plate as described previously [5]. The plates were then left at the bench with the cover up for 2 h in order to absorb the fluid. Colony counting was performed on plates containing 10–100 colonies. The PAE was calculated as T-C, where T was the time necessary for the number of viable organisms in the test culture to increase by $1 \log_{10}$ above the number observed immediately after removal of antibiotics, and C was the time necessary for the number of viable organisms in the untreated culture to increase by $1 \log_{10}$ above the number observed immediately after centrifugation [3].

Determination of PLIE

The culture was prepared in the same way as for PAE determination. Carbapenem (imipenem or meropenem) stock solution plus EDTA was added to the logarithmic phase cultures to yield concentrations of $8 \times \text{MIC}$ or $2 \times \text{MIC}$. After incubation for 2 h at 37°C in a shaking water bath, the antibiotics and EDTA were removed by centrifugation and washing of the pellet twice in PBS. The pellet was then resuspended in the same volume of pre-warmed cation-supplemented MH broth, carbapenem was added at a concentration of $0.25 \times \text{MIC}$ and regrowth of the strain was followed for 8 and 24 h. The untreated control culture was also centrifuged and washed, and carbapenem was added at a concentration of $0.25 \times \text{MIC}$. The 100 μl of sample was applied to the plate with a micropipette and spread over the whole surface of the plate as described previously [5]. The plates were then left at the bench with the cover up for 2 h in order to absorb the fluid. The PLIE was calculated as T-C, where T was the time necessary for the number of viable organisms in the test culture to increase by $1 \log_{10}$ above the number observed immediately after removal of antibiotics, and C was the time necessary for the number of viable organisms in the untreated control culture containing carbapenem after centrifugation to increase by $1 \log_{10}$ above the number observed immediately after centrifugation [3]. The PAE and PLIE were assayed three times.

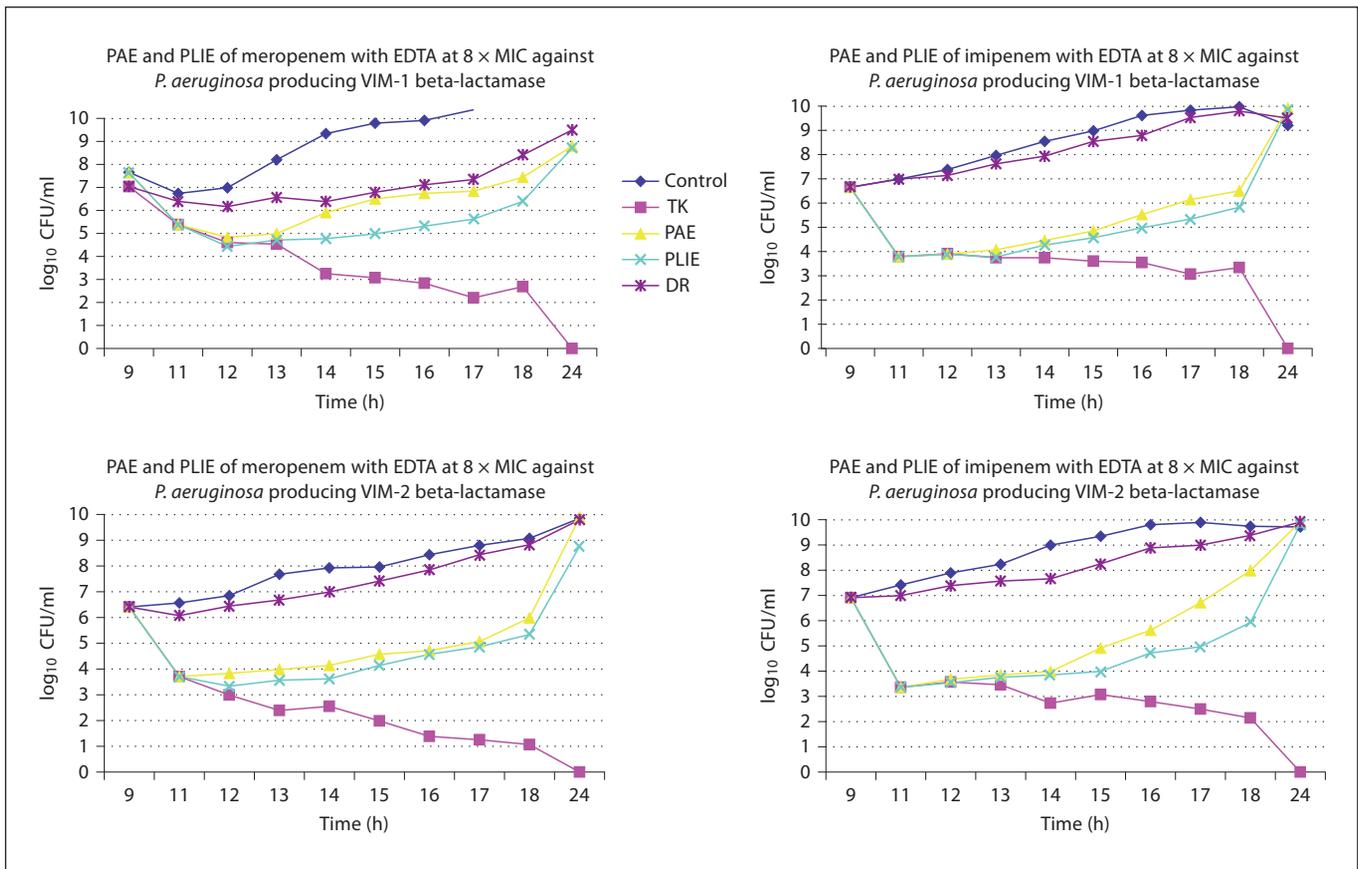


Fig. 1. PAE and PLIE carbapenems combined with EDTA at $8 \times$ MIC against *P. aeruginosa* strains harboring MBLs. Mean values and SDs of three experiments are shown. TK = Time kill curve: the test culture was exposed to $8 \times$ MIC of imipenem or meropenem combined with EDTA. PAE: the test culture was exposed to $8 \times$ MIC of carbapenem and EDTA for 2 h after which both com-

pounds were removed. PLIE: the test culture was exposed to $8 \times$ MIC of carbapenem and EDTA for 2 h after which both compounds were removed and only carbapenem added at concentration $0.25 \times$ MIC. DR (delay of regrowth): The untreated culture was exposed to $0.25 \times$ MIC of carbapenem.

Results

Susceptibility Testing

MICs are shown in table 1. The MIC of imipenem alone was 512 mg/l for both strains and was reduced by EDTA to 16 mg/l for the VIM-1 and 8 mg/l for the VIM-2 strain. The MIC of meropenem alone for VIM-1 was 512 mg/l and was decreased by EDTA to 16 mg/l, whereas the VIM-2 strain had an MIC of meropenem alone of 256 mg/l and combined with EDTA of 8 mg/l. The MBCs of both carbapenems alone exceeded 1,024 mg/l whereas the values in combination with EDTA were 3–4 dilutions higher than the MICs shown in table 1.

Time-Kill Curves

After 8 h of exposure to $8 \times$ MIC of carbapenem + EDTA there were no surviving bacteria in most cases (fig. 1). When cultures were treated with $2 \times$ MIC of carbapenem and EDTA, the viable counts were reduced from 2 to 3 \log_{10} (fig. 2).

Determination of PAE and PLIE

The duration of PAE with meropenem combined with EDTA at $8 \times$ MIC was longer against both the VIM-1 and VIM-2 producers (2.07 ± 0.47 h and 3.06 ± 0.28 h, respectively) than that of imipenem with EDTA on VIM-1- and VIM-2-positive strains (1.44 ± 0.24 h and 1.75 ± 0.55 h, respectively). PLIE after exposure to meropenem combined with EDTA at $8 \times$ MIC lasted 1.86 ± 0.3 h

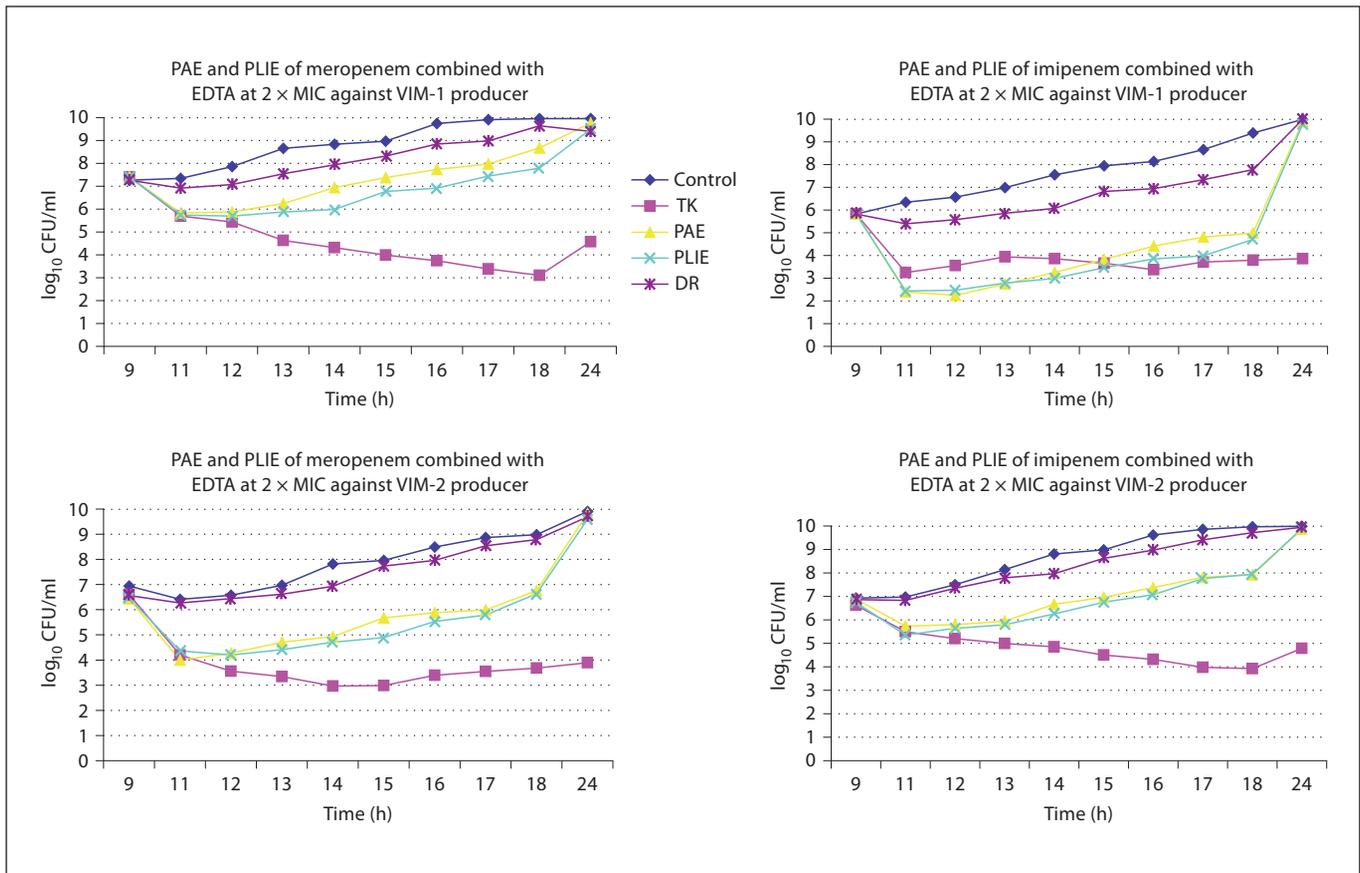


Fig. 2. PAE and PLIE of carbapenems with EDTA at $2 \times$ MIC against *P. aeruginosa* strains harboring MBLs. Mean values and SDs of three experiments are shown. TK = Time kill curve: the test culture was exposed to $8 \times$ MIC of imipenem or meropenem combined with EDTA. PAE: the test culture was exposed to $2 \times$ MIC of carbapenem and EDTA for 2 h after which both com-

pounds were removed. PLIE: the test culture was exposed to $2 \times$ MIC of carbapenem and EDTA for 2 h after which both compounds were removed and only carbapenem added at concentration $0.25 \times$ MIC. DR (delay of regrowth): the untreated culture was exposed to $0.25 \times$ MIC of carbapenem.

against VIM-1 and 1.80 ± 0.15 h against VIM-2 producer. Imipenem with EDTA at higher concentrations induced PLIE of 1.52 ± 0.44 h against the VIM-1-positive strain and 1.26 ± 0.21 h against the VIM-2-positive strain (table 2). PAE and PLIE induced with $8 \times$ MIC is shown in figure 1 (one example of each experiment is shown, not the mean value). At higher carbapenem concentrations, PAEs of both carbapenems were slightly longer for VIM-2 than for VIM-1 producers in contrast with PLIE which was shorter for the VIM-2 compared with the VIM-1-positive strain.

The duration of both PAE and PLIE after exposure to a combination of carbapenem with EDTA at twice the MIC was significantly shorter than following exposure to concentrations of $8 \times$ MIC. At lower concentrations, me-

roperenem with EDTA induced slightly longer PAE (1.18 h for VIM-1, 0.86 h for VIM-2) and PLIE (1.2 h for VIM-1, 1.14 h for VIM 2) than imipenem with EDTA (0.64 h for VIM-1, 0.85 h for VIM-2 and 0.66 h for VIM-1 and 0.71 h for VIM-2, respectively) (table 2). PAE and PLIE induced with $2 \times$ MIC is shown in figure 2 (one example curve of each experiment is shown, not the mean value and SD of three experiments).

Discussion

Several studies have demonstrated in vitro PLIE for amoxicillin/clavulanate with different bacteria producing plasmidic or chromosomal β -lactamases and of am-

Table 1. Minimum inhibitory (MIC) and minimum bactericidal concentrations (MBC) of carbapenems alone and in combination with EDTA against *P. aeruginosa* strains producing MBLs

Strain	MIC				MBC			
	IMI	IMI+EDTA	MEM	MEM+EDTA	IMI	IMI+EDTA	MEM	MEM+EDTA
<i>P. aeruginosa</i> VIM-1	512	16	512	16	>1,024	256	>1,024	128
<i>P. aeruginosa</i> VIM-2	512	8	256	8	>1,024	64	>1,024	128

IMI = Imipenem; MEM = meropenem.

Table 2. PAE and PLIE of imipenem and meropenem combined with EDTA at $8 \times \text{MIC}$ and $2 \times \text{MIC}$ against *P. aeruginosa* strains harboring MBLs

Strain	PAE (h)		PLIE (h)	
	IMI+EDTA ($8 \times \text{MIC}$)	MEM+EDTA ($8 \times \text{MIC}$)	IMI+EDTA ($8 \times \text{MIC}$)	MEM+EDTA ($8 \times \text{MIC}$)
<i>P. aeruginosa</i> VIM-1	1.44 ± 0.24	2.07 ± 0.47	1.52 ± 0.44	1.86 ± 0.3
<i>P. aeruginosa</i> VIM-2	1.75 ± 0.52	3.06 ± 0.28	1.26 ± 0.21	1.80 ± 0.15
Strain	PAE (h)		PLIE (h)	
	IMI+EDTA ($2 \times \text{MIC}$)	MEM+EDTA ($2 \times \text{MIC}$)	IMI+EDTA ($2 \times \text{MIC}$)	MEM+EDTA ($2 \times \text{MIC}$)
<i>P. aeruginosa</i> VIM-1	0.64 ± 0.22	1.18 ± 0.18	0.66 ± 0.39	1.2 ± 0.36
<i>P. aeruginosa</i> VIM-2	0.85 ± 0.55	0.86 ± 0.32	0.71 ± 0.41	1.14 ± 0.44

Mean values and SDs of three experiments are shown. IMI = Imipenem; MEM = meropenem.

picillin/sulbactam against ESBL producing *K. pneumoniae* and *E. coli*. To our knowledge, this is the first study in which PLIE of carbapenems combined with metal chelators has been shown on *P. aeruginosa* strains producing MBLs. The PLIE identified here clearly contributed to the delay in growth observed with the strains tested. Meropenem combined with EDTA induced longer PAE against MBL-positive *P. aeruginosa* strains than imipenem combined with EDTA. There were no significant differences in duration of PAE and PLIE between VIM-1 and VIM-2 producers, although they were slightly longer for the VIM-2-positive strain. This could be due to the slightly different kinetic properties of VIM-1 and VIM-2 MBLs towards carbapenems resulting in different hydrolytic efficiencies, as described previously [15]. Imipenem and meropenem are hydrolyzed more efficiently by VIM-2 compared to VIM-1 MBL (30- and 10-fold). Relatively high SDs in some experiments are probably due to the differences in duration of PAEs in three experiments in spite of the same experimental conditions. The concentration of EDTA used in this study was 0.4 mM because

previous studies have shown that it is sufficient to inhibit MBLs without exerting toxicity on the *P. aeruginosa* strains [16]. The concentrations of carbapenems between $8 \times \text{MIC}$ and $2 \times \text{MIC}$ correspond to the serum concentration of carbapenems measured in vivo during i.v. perfusions [17]. The duration of both PAE and PLIE was concentration dependent. Higher carbapenem concentrations produced longer postexposure effects. However, there were no significant differences in the duration of PAE and PLIE between the two carbapenems. Meropenem displayed slightly longer PAEs and PLIEs in most experiments. The mechanism observed in vitro remains hypothetical. After β -lactamase inhibition by carbapenems with EDTA during the pre-exposure phase, followed by the elimination of EDTA, the surviving bacteria may require a 'latency period' to synthesize a sufficient level of β -lactamase. During this period the carbapenem still present can fully exert its antibiotic activity and inhibits bacterial growth until the β -lactamase concentration is again sufficient to hydrolyze carbapenem and allow bacterial regrowth. PLIE enables intermittent dosing sched-

ules of β -lactam/inhibitor combinations such as coamoxiclav or ampicillin/sulbactam because the inhibition of β -lactamases continues after inhibitor concentrations fall below therapeutic levels [3–5]. The results of this study do not have any clinical relevance so far, since metal chelators such as EDTA are not used as therapeutic agents due to their toxicity, but it is likely to expect that less toxic inhibitors of MBLs are going to be developed in the future. However, the results could be relevant for the characterization of MBLs since they provide information

on the dynamics of MBL inhibition. Susceptibility to inhibitors and kinetics of inhibition are important parameters for the characterization of β -lactamases. The findings merit further work with other MBLs producing *P. aeruginosa* strains such as of the IMP or SPM class.

Acknowledgement

The study was supported by Croatian Ministry of Science, Education and Sport (Grant No. 108-1080114-0015).

References

- Bradford PA: Extended-spectrum β -lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat. *Clin Microbiol Rev* 2001;14:933–951.
- Hanberger H, Lennart EN, Kihlstrom E, Maller R: Postantibiotic effect of β -lactam antibiotics of *Escherichia coli* evaluated by bioluminescence assay of bacterial ATP. *Antimicrob Agents Chemother* 1990;34:102–106.
- Lavigne JP, Bonnet R, Charachon SM, Jourdan J, Caillon J, Sotto A: Post-antibiotic and post- β -lactamase inhibitor effects of ceftazidime plus sulbactam on extended-spectrum β -lactamase producing Gram-negative bacteria. *J Antimicrob Chemother* 2004;53:616–619.
- Thornburn CE, Molesworth SJ, Sutherland R, Rittenhouse S: Postantibiotic and post- β -lactamase inhibitor effect of amoxicillin plus clavulanate. *Antimicrob Agents Chemother* 1996;40:2796–2801.
- Murbach V, Dhoyen N, Kingler H, Jehl F: Evidence for a true post- β -lactamase-inhibitor effect of clavulanic acid against *Klebsiella pneumoniae* and *Haemophilus influenzae*. *Clin Microbiol Infect* 2001;7:661–665.
- Giamarellos-Bourboulis EJ, Kentepozidis N, Antonopoulou A, Plachouras D, Tsaganos T, Giamarellou H: Postantibiotic effect of antimicrobial combinations on multidrug-resistant *Pseudomonas aeruginosa*. *Diagn Microbiol Infect Dis* 2005;51:113–117.
- Boswell FJ, Andrews JM, Wise R: Postantibiotic effect of trovofloxacin on *Pseudomonas aeruginosa*. *J Antimicrob Chemother* 1997;39:811–814.
- Bustamante CI, Drusano GL, Tatem BA, Standford HC: Postantibiotic effect of imipenem on *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 1984;36:678–682.
- Ferrara A, Santos C, Cibro M: Postantibiotic effect of meropenem in combination with gentamicin or sparfloracin on Gram-positive and Gram-negative organisms. *Clin Microbiol Infect* 1998;4:431–434.
- Nordmann P, Poirel L: Emerging carbapenemases in Gram-negative aerobes. *Clin Microbiol Infect* 2002;8:321–331.
- Bonfiglio G, Marchetti F: In vitro activity of ceftazidime, cefepime and imipenem on 1,005 *Pseudomonas aeruginosa* clinical isolates either susceptible or resistant to β -lactams. *Chemotherapy* 2000;46:229–234.
- Walsh F, Bracher S, Turner P, Amyes S: Preferential selection of IMP and VIM metallo- β -lactamases by imipenem in *Pseudomonas aeruginosa*. *Chemotherapy* 2007;53:407–409.
- Lee K, Lim YS, Yong D, Yum JH, Chong Y: Evaluation of the Hodge test and the imipenem-EDTA test and the imipenem-EDTA double-disk synergy test for differentiating metallo- β -lactamase-producing isolates of *Pseudomonas* spp. and *Acinetobacter* spp. *J Clin Microbiol* 2003;41:4623–4629.
- National Committee for Clinical Laboratory Standards: Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically, ed 5. Approved standard M7-A5. Wayne, NCCLS, 2000.
- Docquier JD, Brasseur JL, Galleni M, Amicisante G: On functional and structural heterogeneity of VIM-type metallo- β -lactamases. *J Antimicrob Chemother* 2003;51:257–266.
- Migliavacca R, Docquier JD, Mugnaioli C, Amicosante G, Daturi R, Lee K, Rossolini GM, Pagani L: Simple microdilution test for detection of metallo β -lactamase production in *Pseudomonas aeruginosa*. *J Clin Microbiol* 2002;40:4388–4390.
- Wiseman LR, Wagstaff AJ, Brogden RN, Bryson HM: Meropenem: a review of its antibacterial activity, pharmacokinetic properties and clinical efficacy. *Drugs* 1995;50:73–101.

© Free Author Copy - for personal use only

PLEASE NOTE THAT ANY DISTRIBUTION OF THIS ARTICLE WITHOUT WRITTEN CONSENT FROM S. KARGER AG, BASEL IS A VIOLATION OF THE COPYRIGHT. Upon request a written permission to distribute the PDF file will be granted against payment of a permission fee depending on the number of accesses required. Please contact Karger Publishers, Basel, Switzerland at permission@karger.ch