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NOTE

Efficacies of calcium–EDTA in combination with imipenem in a murine model of sepsis caused by *Escherichia coli* with NDM-1 β-lactamase

Ayumi Yoshizumi · Yoshikazu Ishii · David M. Livermore · Neil Woodford · Soichiro Kimura · Tomoo Saga · Sohei Harada · Keizo Yamaguchi · Kazuhiro Tateda

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Abstract We evaluated the efficacy of ethylenediamine-*N,N,N′,N′*-tetraacetic acid, disodium calcium salt (Ca-EDTA), as an inhibitor for New Delhi metallo-β-lactamase-1 (NDM-1) in vitro antibiotic susceptibility and in a mouse model of sepsis caused by *Escherichia coli*. Ca-EDTA drastically reduced the MICs of carbapenems for all NDM-producing bacteria [imipenem (IPM) ≤1–2 μg/ml; meropenem (MEPM) ≤1–4 μg/ml]. In the neutropenic murine model of sepsis, the bacterial burden was further reduced by combination therapy using imipenem/cilastatin sodium (IPM/CS) and Ca-EDTA to 2.3×10^3 CFU/liver, compared with 2.9×10^4 CFU/liver for IPM/CS alone. These data demonstrated the possibility of Ca-EDTA for clinical applications. In our understanding, this is the first report examining the effect of Ca-EDTA on a mouse sepsis model caused by NDM-1-producing bacteria.

Keywords Antibiotic resistance · NDM-1 · Metallo-β-lactamase inhibitor · Calcium-EDTA

A. Yoshizumi · Y. Ishii (✉) · S. Kimura · T. Saga · S. Harada · K. Yamaguchi · K. Tateda
Department of Microbiology and Infectious Diseases, Faculty of Medicine, Toho University School of Medicine, Tokyo 143-8540, Japan
e-mail: yishii@med.toho-u.ac.jp

D. M. Livermore · N. Woodford
Antibiotic Resistance Monitoring and Reference Laboratory, Health Protection Agency, Colindale, London, UK

D. M. Livermore
Norwich Medical School, University of East Anglia, Norwich, UK

NDM-1 (New Delhi metallo-β-lactamase-1) is a recently discovered transferable molecular class B (zinc metallo-β)-lactamase. It hydrolyzes and inactivates all β-lactams except aztreonam (AZT). Its encoding gene, $bla_{\text{NDM-1}}$, has been widely identified in clinical *Enterobacteriaceae* and *Acinetobacter baumannii* isolates internationally, but often with epidemiological links to India and Pakistan [1–4]. Its diverse encoding plasmids have a wide host range and are readily self-transmissible in the laboratory; they have been found in remotely related gram-negative groups including the *Enterobacteriaceae*, *Aeromonas*, *Pseudomonas*, *Stenotrophomonas*, and *Vibrio cholerae* [5]. Most *Enterobacteriaceae* and *A. baumannii* with NDM-1 enzyme are resistant to wide ranges of both β-lactam and non-β-lactam antibiotics and often are more comprehensively resistant than isolates with other carbapenemases such as KPC and VIM enzymes [3, 4, 6].

Several in vivo studies have been reported on treatments directed against bacteria producing NDM-1 β-lactamase. A combination of colistin plus tigecycline was active against *Klebsiella pneumoniae* and *Escherichia coli* with the enzyme [7]. However, these are not ideal drugs: polymyxins are nephrotoxic, and tigecycline is unsuitable for use in the urinary tract and has questionable efficacy in nosocomial pneumonia. Many *E. coli* with the NDM-1 enzyme are susceptible to fosfomycin in vitro; however, it is unsuitable for monotherapy except in the urinary tract and is less active against many *K. pneumoniae* [6].

Ethylenediamine-*N,N,N′,N′*-tetraacetic acid (EDTA) is a metal chelator with direct antimicrobial activity and an ability to potentiate other classes of antibiotics, largely by disrupting the outer membrane [8, 9]. It has also the potential to detoxify and neutralize bacterial toxins/enzymes [10, 11]. For metallo β-lactamase (MBL) diagnosis, IPM-EDTA synergy such as Etest is usually used in

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clinical laboratories. Despite these beneficial effects, the toxicity of EDTA has prevented its widespread clinical use. An important exception is ethylenediamine-*N,N,N′,N′*-tetraacetic acid, disodium calcium salt (Ca-EDTA), a complex of EDTA and the calcium ion. This compound was created as an injectable chelator with reduced toxicity and is approved and commercialized for the treatment of lead poisoning in Japan [8, 12]. Aoki et al. previously reported the efficacy of Ca-EDTA as an inhibitor of metallo-enzymes, such as IMP-1 β-lactamase and proteases, in a mouse model of *Pseudomonas aeruginosa* pneumonia [13]. In the present study, we investigated the in vitro synergy between Ca-EDTA and IPM for bacteria with NDM-1 enzyme and evaluated the efficacy of Ca-EDTA as an inhibitor for NDM-1 enzyme in a mouse model of *E. coli* sepsis.

In vitro susceptibilities to imipenem monohydrate (IPM), meropenem trihydrate (MEPM), amikacin hydrate (AMK), AZT, and ciprofloxacin (CPFX) (Sigma-Aldrich Japan, Tokyo, Japan) were determined by the broth microdilution method of the Clinical and Laboratory Standards Institute (CLSI) [14] in the presence or absence of 32 μg/ml Ca-EDTA (Dojindo Laboratory, Kumamoto, Japan) for isolates with NDM-1 enzyme, comprising *K. pneumoniae* NCTC13443 [15] and TUM10700 and *Escherichia coli* TUM10701 and TUM10702. The concentration of Ca-EDTA for MIC was 32 μg/ml, as described previously [13]. These in vitro tests showed that the addition of Ca-EDTA reduced the MICs of carbapenems in all NDM-1 positive strains at least 32 fold; the MICs of IPM and MEPM were reduced from ≥64 to ≤2 and ≤4 μg/ml, respectively (Table 1). In contrast, no potentiating effects of Ca-EDTA were observed for the MICs of AMK, CPFX, and AZT.

An acute lethal septicemia was induced in 20 mice by intraperitoneal (i.p.) injection of approximately 2.7×10^7 colony-forming units (CFU) of *E. coli* TUM10702 per mouse. Two hours after infection the mice were split into groups of four and subcutaneously administered (i) saline as a control, (ii) Bleian (Nisshin Pharmaceutical, Yamagata, Japan) as an injectable form of Ca-EDTA, (iii) imipenem/cilastatin sodium (IPM/CS, Tienam; MSD, Tokyo, Japan), or (iv) Ca-EDTA plus IPM/CS. All animal experiments were performed according to the instructions of the Toho University Animal Center (Permission No. 11-53-54). The dosage of Ca-EDTA was 200 mg/kg as described previously [13], and the concentration of IPM/CS was 25 mg/kg, which was determined by the preliminary examination (data not shown). Four hours after the induction of sepsis, the mice were killed and blood and liver samples were taken for analysis. Livers were homogenized on ice in 2 ml sterile saline using a tissue homogenizer (Omni International, NW Kennesaw, GA, USA). The liver homogenates (10 μl) and blood samples (30 μl) were inoculated onto Mueller–Hinton agar after serial 1:10 dilution. The plates were then incubated overnight at 35 °C before counting the colonies that grew. Differences in the number of bacteria recovered from the mice that received IPM/CS monotherapy and those that received IPM/CS plus Ca-EDTA were assessed using the two-tailed Student's *t* test, with $P < 0.05$ considered to indicate a significant difference.

The mouse studies showed that bacterial burden was significantly reduced ($P < 0.05$) by IPM/CS plus Ca-EDTA to 2.1×10^4 CFU/liver, compared with 7.5×10^5 CFU/liver for IPM/CS alone, Ca-EDTA alone, and for the saline control. The burden in blood was reduced by IPM/CS plus Ca-EDTA to 4.0×10^3 CFU/ml blood, compared with 6.1×10^4 CFU/ml blood for IPM/CS alone, a difference that narrowly failed to meet statistical significance ($P = 0.08$). To evaluate the efficacy of Ca-EDTA with IPM/CS in a more serious sepsis model, neutropenia was induced by the subcutaneous injection of cyclophosphamide (Endoxan; Shionogi, Osaka, Japan) at 150 mg/kg on day −4 and 100 mg/kg on day −2 preinfection. Septicemia was induced

Table 1 Effects of Ca-EDTA on minimum inhibitory concentration (MIC) of imipenem, meropenem, aztreonam, amikacin, and ciprofloxacin

Antibiotics	Ca-EDTA[a]	MIC (μg/ml)			
		Klebsiella pneumoniae NC13443	*K. pneumoniae* TUM10700	*Escherichia coli* TUM10701	*E. coli* TUM10702
imipenem	−	64	64	64	512
	+	1	2	1	2
meropenem	−	256	256	256	256
	+	4	4	1	2
aztreonam	−	>512	>512	>512	>512
	+	>512	>512	>512	>512
amikacin	−	>64	>64	>64	>64
	+	>64	>64	>64	>64
ciprofloxacin	−	>64	32	64	16
	+	>64	16	32	8

[a] Ethylenediamine-*N,N,N′,N′*-tetraacetic acid, disodium calcium salt