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ORIGINAL ARTICLE

Synergy of imipenem/colistin methanesulfonate combinations against imipenem-nonsusceptible multidrug-resistant *Acinetobacter baumannii*

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Background: The optimal combination ratio of imipenem to colistin methanesulfonate (CMS) against imipenem-nonsusceptible multidrug-resistant *Acinetobacter baumannii* (INS-MDRAB) has not been determined in previous studies. To provide an alternative therapeutic option for clinical INS-MDRAB isolates, we investigated whether clinically achievable serum concentrations of CMS in combination with imipenem enhance the *in vitro* activity of imipenem against the INS-MDRAB isolates.
Materials and methods: Fifty-nine INS-MDRAB isolates with imipenem minimal inhibitory concentration (MIC) values of $\geq$8 mg/L were selected randomly from the Clinical Microbiology Laboratory at a university-affiliated medical center between July 1998 and May 2005. The *in vitro* activity of imipenem among these 59 clinical isolates was explored via serial two-fold dilutions containing a range of imipenem concentration from 0.125 mg/L to 256 mg/L, in combination with two fixed CMS concentrations at 0.5 mg/L and 1 mg/L. Genotype classification was performed using the pulsed-field gel electrophoresis method and infrequent-restriction-site polymerase chain reaction.

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Results: A significant reversal of imipenem resistance (i.e., MICs $\leq$ 4 mg/L) was observed in 34 (57.6%) isolates and 44 (74.6%) isolates with the tests of CMS concentrations at 0.5 mg/L and 1 mg/L, respectively ($p = 0.041$). Genotype 1 was predominant (43 isolates, 72.9%) with imipenem resistance reversal rates of 51.2% and 79.1% ($p = 0.004$) in the tests of CMS at 0.5 mg/L and 1 mg/L, respectively.
Conclusion: The synergy of imipenem/CMS against INS-MDRAB was significantly better for the CMS concentration at 1 mg/L than that at 0.5 mg/L, especially in our predominant clone. Our results provided insightful information for treating INS-MDRAB infections in clinical practice.

Introduction

Multidrug-resistant *Acinetobacter baumannii* (MDRAB) is often defined as *A. baumannii* resistant to three or more of the following classes of antimicrobials: aminoglycosides, antipseudomonal fluoroquinolones, antipseudomonal carbapenems, extended-spectrum cephalosporins, antipseudomonal penicillins plus beta-lactamase inhibitors, sulbactam, polymyxins, and tigecycline.[1,2] Carbapenem-nonsusceptible *A. baumannii* has emerged as an important nosocomial pathogen in the hospital setting. Most of them were MDRAB with resistance to multiple antibiotics and *in vitro* susceptibility to only a few drugs, such as colistin.[3,4] Colistin methanesulfonate (CMS), a parenteral formulation of colistin, was gradually abandoned by clinicians in the 1960s and 1970s due to frequently reported dose-dependent side effects, such as nephrotoxicity and neurotoxicity. In recent years, CMS has been reintroduced in clinical practice due to limited therapeutic options for carbapenem-nonsusceptible MDRAB.[4]

Either *in vivo* or *in vitro*, CMS is readily hydrolyzed to colistin, which was considered an active form of the drug with a concentration-dependent bactericidal effect against *Acinetobacter* species.[4,5] However, in recent pharmacokinetic studies, the achieved plasma colistin concentrations were found to be in the range of only 1—4 mg/L after intravenous administration of CMS in humans,[6–8] which suggested that the currently recommended CMS dosage might be suboptimal for antibacterial effect in treating pathogens with higher minimal inhibitory concentrations (MICs).[4,5,8] Dose escalation may maximize CMS efficacy against MDRAB but, at the same time, may increase dose-dependent toxicities.[4]

Imipenem/CMS combination can be an alternative therapeutic option for infections due to imipenem-nonsusceptible MDRAB (INS-MDRAB),[9,10] and a high serum level of colistin to achieve the concentration-dependent bactericidal effect may not be necessary under such a combination. Imipenem/CMS combination can improve the activity of imipenem against INS-MDRAB via a synergistic or additive effect with a lower serum level of colistin, reduce the dose-dependent toxicities, and prevent the emergence of colistin heteroresistance during colistin monotherapy.[9–11] In clinical practice, it is difficult to provide the optimal combination ratio of imipenem to CMS for individual cases, according to previous synergy study.[3] To provide an alternative therapeutic option for clinical INS-MDRAB isolates, we conducted a study to examine the *in vitro* activity of imipenem in combination with CMS at fixed concentrations of 0.5 mg/L and 1 mg/L, which were achievable serum CMS or colistin concentrations in prior studies.[6–8] The aim of this study was to investigate the imipenem resistance reversal in clinical INS-MDRAB isolates using imipenem in combination with CMS at clinically achievable concentrations.

Materials and methods

Bacterial isolates and genotype classification

The Chang Gung Memorial Hospital at Linkou is a 3715-bed university-affiliated medical center with 308 intensive-care-unit beds in northern Taiwan. A central microbiology laboratory is responsible for processing all clinical specimens. For the surveillance of hospital epidemiology, *A. baumannii* clinical isolates were collected in the first week of each month between July 1998 and May 2005, and were stored in skimmed milk at −70°C in the central microbiology laboratory. A total of 2475 *A. baumannii* isolates were stored during this period. *A. baumannii* was identified by the Gram's stain and conventional biochemical tests.[12] Briefly, the identification of clinical isolates as the genus *Acinetobacter* is based on the following properties: aerobic, Gram-negative, nonmotile coccobacillary rods with a non-fermentative, catalase-positive, and oxidase-negative reaction. *Acinetobacter* species with glucose-oxidizing nonhemolytic characteristics are classified as *A. baumannii*.

The antibiotic disks (Becton Dickinson Microbiology Systems, Cockeysville, MD, USA) for the susceptibility testing of *A. baumannii* included gentamicin, amikacin, ceftazidime, cefepime, aztreonam, ciprofloxacin, piperacillin, and imipenem. The disk diffusion susceptibility to all tested antibiotics was based on the Clinical and Laboratory Standards Institute (CLSI) criteria.[13] Of the 2475 stored *A. baumannii* isolates, 1196 were MDRAB isolates with a variable susceptibility to imipenem but resistance to all the other classes of antibiotics mentioned above.[2] Among the 1196 MDRAB isolates (including imipenem-susceptible and imipenem-nonsusceptible strains) 90 were selected randomly and 59 of them were INS-MDRAB, all of which were included in this study.

Genotype classification was performed for these 59 isolates using the pulsed-field gel electrophoresis (PFGE) method and infrequent-restriction-site polymerase chain reaction (IRS-PCR) that had been used in previous studies.[14,15] Band patterns were analyzed comparatively, according to the criteria of Tenover et al.[14]

Determination of combination effects of antimicrobial agents

Standard powders of imipenem and CMS were obtained from Merck (Rahway, NJ, USA) and Sigma-Aldrich (St. Louis, MO, USA), respectively. The MICs of imipenem and CMS for the 59 INS-MDRAB isolates were determined by the broth microdilution method, according to the CLSI criteria.[13] The MIC value for imipenem nonsusceptibility was ≥8 mg/L.[13] The synergy study for IPM/CMS combinations was performed for six isolates selected randomly from the 59 INS-MDRAB isolates, using the checkerboard method.[16] Serial two-fold dilutions for imipenem (ranging from 0.125 mg/L to 256 mg/L) and CMS (ranging from 0.125 mg/L to 8 mg/L) were mixed in the cation-supplemented Müeller–Hinton broth (Oxoid Ltd., Hampshire, England). Each well contained 0.1 mL of individual antimicrobial combinations or broth controls. The final bacterial concentration after inoculation was 5×10^5 colony-forming units/mL (CFU/mL). After 24 hours of incubation at 35°C, the MIC was determined to be the minimal concentration at which there was no visible growth. Quality control was performed in each experiment using reference strains, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853.[13] The synergistic effects of the combination of two antimicrobials were based on a calculation of the fractional inhibitory concentration index (FICI) for each drug pair. The fractional inhibitory concentration (FIC) of each antimicrobial agent was calculated using the following equations: FIC of imipenem = MIC of imipenem in combination/MIC of imipenem alone, and the FIC of CMS = MIC of CMS in combination/MIC of CMS alone. FICI is the summation of FIC values for the drugs in combination.[16] FICI results for each combination were interpreted as follows: synergistic, FICI ≤ 0.5; partially synergistic, 0.5 < FICI < 1; additive, FICI = 1; indifferent, 1 < FICI < 4; and antagonistic, FICI ≥ 4.[16] *In vitro* susceptibility change of imipenem in combination with CMS against all 59 INS-MDRAB isolates was investigated with serial two-fold dilutions for imipenem ranging from 0.125 mg/L to 256 mg/L and CMS at fixed concentrations of 0.5 mg/L and 1 mg/L.

Statistical methods

All statistical analyses were performed using the SPSS for Windows (version 15.0; SPSS Inc., Chicago, IL, USA). Categorical variables were compared using the χ^2 test or Fisher exact test, as appropriate. Paired samples were compared using the McNemar's test. All tests were two tailed, and $p < 0.05$ was considered significant.

Results

The results of the synergy study for IPM/CMS combinations against six randomly selected INS-MDRAB isolates are shown in Table 1. Among these six isolates, five had synergistic effects with FICI values ranging from 0.25 to 0.5 and one had indifferent effect with a FICI value of 0.563. The changes of imipenem MICs of the 59 isolates tested with imipenem in combination with CMS at fixed concentrations of 0.5 mg/L and 1 mg/L are shown, respectively, in Tables 2 and 3. In the test with CMS concentration at 0.5 mg/L, the MIC_{50} and MIC_{90} values of imipenem alone were 8 mg/L and 16 mg/L, respectively (Table 2). Among the 59 isolates tested with imipenem/CMS combination, 18 (30.5%) had at least a four-fold decrease in imipenem MICs and 34 (57.6%) had a reversal of imipenem resistance with the change of imipenem MICs from ≥8 to ≤4 mg/L (Table 2). In the test with CMS at concentration at 1 mg/L, the MIC_{50} and MIC_{90} values of imipenem alone were 16 mg/L and 32 mg/L, respectively (Table 3). Among the 59 isolates tested with the combinations, 34 (57.6%) had at least a four-fold decrease in imipenem MICs and 44 (74.6%) had imipenem MICs ≤ 4 mg/L (Table 3). Most isolates with reversal of imipenem resistance had at least a four-fold decrease in MICs (33/44, 75%; Table 3). The imipenem/CMS combination with the CMS concentration of 1 mg/L had a higher reversal rate of imipenem resistance than that with the CMS concentration of 0.5 mg/L (74.6% vs. 57.6%, $p = 0.041$; Table 4).

Table 1 Results of the synergy study for imipenem/CMS combination against the six randomly selected INS-MDRAB isolates

Isolate code	Genotype	FICI value	Interpretation
615	Non-1	0.563	Indifference
622	Non-1	0.375	Synergy
623	Non-1	0.5	Synergy
1456	1	0.25	Synergy
1468	1	0.25	Synergy
1523	1	0.266	Synergy

CMS = colistin methanesulfonate; FICI = fractional inhibitory concentration index; INS-MDRAB = imipenem-nonsusceptible multidrug-resistant *Acinetobacter baumannii*.

Twenty-two INS-MDRAB isolates with the CMS MIC of 1 mg/L were tested with imipenem in combination with CMS at concentrations of 0.5 mg/L and 1 mg/L, and the reversal rates of imipenem resistance were 63.6% and 81.8%, respectively ($p = 0.219$). Of 37 isolates with CMS MICs ≥ 2 mg/L, 34 had a MIC of 2 mg/L, and the MICs of the remaining three were 4 mg/L, 8 mg/L, and >8 mg/L, respectively. Among these 37 isolates, the reversal rates of imipenem resistance tested with imipenem in combination with CMS at concentrations of 0.5 mg/L and 1 mg/L were 54.1% and 70.3%, respectively ($p = 0.180$; Table 4).

The genetic associations of the 59 *A. baumannii* isolates determined by the PFGE method and IRS-PCR were separated into 12 distinct groups. Genotype 1 was the predominant genotype (43/59, 72.9%). The imipenem susceptibility in the test of imipenem combined with various concentrations of CMS in different genotypes is shown in Table 5. A comparison between the genotype 1 isolates and others showed that the genotype 1 had a lower rate of imipenem resistance reversal when tested with a CMS concentration of 0.5 mg/L (51.2% vs. 75.0%, $p = 0.100$) but a higher rate of imipenem resistance reversal when tested with a CMS concentration of 1 mg/L (79.1% vs. 62.5%, $p = 0.312$). Compared with the genotype 1 isolates tested with a CMS concentration of 0.5 mg/L, there was a 27.9% increase in the imipenem resistance reversal rate when the

Table 2 Imipenem MICs of the 59 INS-MDRAB isolates tested with imipenem alone and the imipenem MIC change of the 59 isolates tested with imipenem in combination with CMS at a fixed concentration of 0.5 mg/L[a]

	IPM MIC (mg/L)	Isolates[b] (n = 59)	IPM MIC change in combination with CMS[c]				
			No change	1/2×	1/4×	1/8×	1/64×
	256	1 (1.7)	1				
	32	3 (5.1)			3		
MIC_{90}	16	19 (32.2)	4	5	6	3	1
MIC_{50}	8	36 (61.0)	12	19	4	1	

[a] Gray area presents the number of INS-MDRAB isolates with IPM MICs ≤ 4 mg/L in IPM/CMS combination.
[b] Data are presented as n (%).
[c] Data are presented as n.
CMS = colistin methanesulfonate; INS-MDRAB = imipenem-nonsusceptible multidrug-resistant *Acinetobacter baumannii*; IPM = imipenem; MIC = minimal inhibitory concentration.

isolates tested with a CMS concentration of 1 mg/L, and the difference was statistically significant (79.1% vs. 51.2%, $p = 0.004$). However, such difference in the isolates of genotype non-1 was not statistically significant (75.0% vs. 62.5%, $p = 0.625$; Table 5).

Discussion

In this study, the imipenem/CMS combination improved the *in vitro* activity of imipenem for most of the studied INS-MDRAB isolates. The imipenem MIC ≤4 mg/L was thought to be *in vitro* susceptible. The decrease of imipenem MICs to ≤4 mg/L was noted in most cases, and the synergy of the combination with CMS at 1 mg/L had a better effect than that at 0.5 mg/L, especially in the isolates of genotype 1, which was the predominate genotype in our isolates. The rationale of the combination therapy in *A. baumannii* infections is to reduce the emergence of resistant isolates and strengthen the efficacy of treatment itself. However, there are no well-designed clinical trials comparing treatment regimens for MDRAB infections. This study provided an alternative therapeutic strategy in treating INS-MDRAB infections: imipenem combined with CMS at a fixed concentration of 1 mg/L, may achieve a reversal of imipenem resistance in up to 74.6% of these MDRAB isolates initially with full or intermediate imipenem resistance.

The aim of our study is to provide a feasible alternative therapeutic regimen, based on the data from the investigation of a tertiary teaching hospital, but our results may not be applicable to all hospital settings due to variations in hospital epidemiology and antibiotic resistance patterns. Our isolates were collected from a single institution, and genotype classification was performed by the PFGE method and IRS-PCR. It showed that one predominant clone existed in our institution, which had a significant decrease in imipenem MICs while tested with imipenem/CMS combinations. The PFGE method and IRS-PCR have been used in epidemiologic studies of numerous *A. baumannii* outbreaks and are currently regarded as the gold standards for epidemiologic typing.[15,17] However, its poor interlaboratory reproducibility may limit its role in the comparison of results between different laboratories. Multilocus sequence typing is a more discriminative method of typing *A. baumannii* for interlaboratory comparisons.[18]

We cannot determine which of colistin or CMS improved the *in vitro* effect of imipenem. The response of imipenem resistance reversal in this study may even be underestimated. The reasons are as follows. First, the real concentrations of CMS or colistin would be ≤0.5 mg/L or 1 mg/L during the testing with CMS hydrolysis, and the response in this study was achieved with a lower concentration of CMS or colistin than the concentration we reported. Second, the response of imipenem resistance reversal was

Table 3 Imipenem MICs of the 59 INS-MDRAB isolates tested with imipenem alone and the imipenem MIC change of the 59 isolates tested with imipenem in combination with CMS at a fixed concentration of 1 mg/L[a]

	IPM MIC (mg/L)	Isolates[b] (n = 59)	IPM MIC change in combination with CMS[c]						
			No change	1/2×	1/4×	1/8×	1/16×	1/32×	1/128×
	256	1 (1.7)	1						
	64	2 (3.4)						2	
MIC_{90}	32	4 (6.8)			1		2	1	
MIC_{50}	16	28 (47.5)	3	7	8	8	1		1
	8	24 (40.7)	3	11	7	2	1		

[a] Gray area presents the number of INS-MDRAB isolates with IPM MICs ≤ 4 mg/L in IPM/CMS combination.
[b] Data are presented as n (%).
[c] Data are presented as n.
CMS = colistin methanesulfonate; INS-MDRAB = imipenem-nonsusceptible multidrug-resistant *Acinetobacter baumannii*; IPM = imipenem; MIC = minimal inhibitory concentration.

Table 4 Change of imipenem susceptibility in the 59 INS-MDRAB isolates tested with imipenem alone and in combination with CMS at various concentrations

Tested antibiotics	Isolates with IPM susceptibility (IPM MIC $\leq$ 4 mg/L[a])		
	CMS MIC = 1 mg/L (n = 22)	CMS MIC$\geq$2 mg/L (n = 37)	Total (n = 59)
IPM alone	0 (0%)	0 (0%)	0 (0%)
IPM + CMS (0.5 mg/L)	14 (63.6%)	20 (54.1%)	34 (57.6%)
IPM + CMS (1 mg/L)	18 (81.8%)	26 (70.3%)	44 (74.6%)

[a] Data are presented as n (%).
CMS = colistin methanesulfonate; INS-MDRAB = imipenem-nonsusceptible multidrug-resistant *Acinetobacter baumannii*; IPM = imipenem; MIC = minimal inhibitory concentration.

dependent on CMS concentrations, i.e., the higher the CMS concentrations, the higher the rates of imipenem resistance reversal. Applying our data in clinical practice does not necessarily depend on the increasing doses of CMS to achieve the concentration-dependent bacterial effect. Most of our INS-MDRAB isolates had high CMS MICs, and the serum concentrations with bactericidal effect might be unachievable from current CMS dose regimens. This study provided a strategy to improve imipenem activity against INS-MDRAB isolates with achievable serum concentrations of colistin or CMS demonstrated in previous studies.[6–8]

In this study, the tests with CMS at concentrations of 0.5 mg/L and 1 mg/L were performed at different times for the 59 clinical INS-MDRAB isolates, and discordant results were reported for imipenem MICs while testing with imipenem alone for each isolate in the two tests. All the variations conformed to the accepted norm of MIC testing (mode $\pm$ 1 dilution), and no isolates were tested as imipenem-susceptible strains (i.e., imipenem MIC $\leq$4 mg/L) in the absence of CMS combination. Compared with the CMS concentration of 0.5 mg/L in the combination, CMS concentration of 1 mg/L achieved a higher rate of at least a four-fold decrease in imipenem MICs (75.0%) among the isolates with imipenem resistance reversal, under higher levels of MIC_{50} and MIC_{90}. This study showed a significant difference in the imipenem susceptibility of the INS-MDRAB isolates between the use of imipenem alone and the imipenem/CMS combination; however, the possibilities for reproducibility errors in an MIC checkerboard could not be completely excluded.

Table 5 Imipenem susceptibility in different genotypes of INS-MDRAB isolates tested with imipenem in combination with CMS at various concentrations

Tested antibiotics	Isolates with IPM susceptibility (IPM MIC $\leq$4 mg/L)[a]	
	Genotype 1 (n = 43)	Genotype non-1 (n = 16)
IPM + CMS (0.5 mg/L)	22 (51.2)	12 (75.0)
IPM + CMS (1 mg/L)	34 (79.1)	10 (62.5)

[a] Data are presented as n (%).
CMS = colistin methanesulfonate; INS-MDRAB = imipenem-nonsusceptible multidrug-resistant *Acinetobacter baumannii*; IPM = imipenem; MIC = minimal inhibitory concentration.

In conclusion, the combination ratio of imipenem to CMS in this study reversed full or intermediate imipenem resistance in most of the INS-MDRAB isolates *in vitro*, and the CMS concentration of 1 mg/L had a significantly better effect than that of 0.5 mg/L, especially in our predominant clone. The results provided insightful information for clinical practice in treating INS-MDRAB infections in our institution. Our study was limited to the *in vitro* antibacterial combination testing; therefore, this combination needs to be interpreted in further clinical studies to delineate its clinical response.

Conflicts of interest

All contributing authors declare no conflicts of interest.

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