In vitro time-kill studies of antimicrobial agents against blood isolates of imipenem-resistant Acinetobacter baumannii, including colistin- or tigecycline-resistant isolates

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The emergence of colistin or tigecycline resistance as well as imipenem resistance in Acinetobacter baumannii poses a great therapeutic challenge. The bactericidal and synergistic effects of several combinations of antimicrobial agents against imipenem-, colistin- or tigecycline-resistant A. baumannii isolates were investigated by in vitro time-kill experiments. Six imipenem-resistant A. baumannii blood isolates were examined in this study, including colistin- and tigecycline-susceptible, colistin-resistant but tigecycline-susceptible, and colistin-susceptible but tigecycline-resistant isolates. Time-kill studies were performed using five antimicrobial agents singly or in combinations (imipenem plus colistin, imipenem plus amoxicillin-sulbactam, colistin plus rifampicin, colistin plus tigecycline, and tigecycline plus rifampicin) at concentrations of 0.5× and 1× their MICs. Only imipenem was consistently effective as a single agent against all six A. baumannii isolates. Although the effectiveness of combinations of 0.5× MIC antimicrobial agents was inconsistent, combination regimens using 1× MIC of the antimicrobial agents displayed excellent bactericidal activities against all six A. baumannii isolates. Among the combinations of 0.5× MIC antimicrobial agents, the combination of colistin and tigecycline showed synergistic or bactericidal effects against four of the isolates. This in vitro time-kill analysis suggests that antimicrobial combinations are effective for killing imipenem-resistant A. baumannii isolates, even if they are simultaneously resistant to either colistin or tigecycline. However, the finding that the combinations of 0.5× MIC antimicrobial agents were effective on only some isolates may warrant further investigation of the doses of combination agents needed to kill resistant A. baumannii.

†These authors contributed equally to this work.

Abbreviation: MDR, multidrug resistant.
INTRODUCTION

Acinetobacter baumannii has emerged as an important nosocomial pathogen, especially in intensive care units (Dijkshoorn et al., 2007). A. baumannii infections may be difficult to treat due to the pathogen’s multidrug resistance. Although carbapenems, including imipenem and meropenem, have been commonly used as the mainstay of treatment for severe A. baumannii infections, carbapenem-resistant isolates have emerged and disseminated worldwide in recent years (Perez et al., 2007). With the exception of polymyxins (such as polymyxin B and colistin) and tigecycline, few alternative therapeutic options are available (Munoz-Price & Weinstein, 2008). However, polymyxin-resistant isolates of A. baumannii have also developed (Li et al., 2006a; Park et al., 2009a), along with tigecycline-resistant isolates (Capone et al., 2008; Park et al., 2009b). Even pandrug-resistant (PDR) A. baumannii isolates, displaying resistance to all antimicrobial agents, including both polymyxins and tigecycline, have recently emerged (Doi et al., 2009; Park et al., 2009c).

A. baumannii isolates cause bloodstream infection, nosocomial-acquired pneumonia or ventilator-associated pneumonia in critically ill patients. Especially those with inappropriate treatment are associated with higher mortality (Falagas et al., 2006). However, the development of new antimicrobial agents to combat A. baumannii infections has been slow. Thus, the use of combinations of two or more agents has drawn attention as an option for treating multidrug-resistant (MDR) A. baumannii infections (Munoz-Price & Weinstein, 2008; Peleg et al., 2008), although the effectiveness of such combinations remains controversial (Moland et al., 2008; Schetz et al., 2007). In addition to increasing eradication efficacy, combination therapy may also help to prevent the emergence of resistant populations (Pachón-Ibáñez et al., 2006). So far, several combinations, such as imipenem and ampicillin-sulbactam, rifampicin and polymyxin B, imipenem and polymyxin B, and colistin and rifampicin, have been reported to be effective in vitro against carbapenem-resistant A. baumannii (Perez et al., 2007; Peleg et al., 2008). However, studies on the effects of these combinations against colistin- or tigecycline-resistant A. baumannii isolates are very limited (Moland et al., 2008).

In this study, we investigated the synergistic and bactericidal effects of combinations of antimicrobial agents against carbapenem-resistant A. baumannii blood isolates that were also resistant to either colistin or tigecycline by in vitro time-kill analysis using a microdilution method.

METHODS

Bacterial isolates. Six representative imipenem-resistant A. baumannii blood isolates that were isolated from intensive care unit patients at four university hospitals in South Korea were included in the present study (Table 1). All were also resistant to meropenem. Two isolates (KRU-A-3 and KHU-4) were resistant to carbapenems, but susceptible to polymyxins and tigecycline (COL-S/TIG-S), two isolates (KCU-4 and SKKU-2) were resistant to polymyxins but susceptible to tigecycline (COL-R/TIG-S), and two isolates (SKKU-8 and KCU-3) were resistant to tigecycline but susceptible to polymyxins (COL-S/TIG-R). All six isolates were resistant to ampicillin-sulbactam. While two isolates (KHU-4 and KCU-4) were resistant to rifampicin, all the others were susceptible to rifampicin.

Determination of MIC. In vitro antimicrobial susceptibility testing was performed by measuring MIC using the broth microdilution method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2010). Fifteen antimicrobial agents were tested: imipenem, meropenem, polymyxin B, colistin, ciprofloxacin, rifampicin, amikacin, ceftepime, ceftriaxone, cefoperazone-sulbactam, ceftazidime, piperacillin-tazobactam, ampicillin-sulbactam, tetracycline and tigecycline. Fresh Mueller–Hinton broth was used for all susceptibility testing. CLSI susceptibility interpretive criteria were used (CLSI, 2010). No breakpoints for rifampicin and tigecycline are available in the CLSI guidelines; therefore CLSI criteria recommended for staphylococci were applied to rifampicin (resistant ≥4 mg l⁻¹), and the criteria of the United States Food and Drug Administration for Enterobacteriaceae were used for tigecycline (intermediate 4 mg l⁻¹; resistant ≥8 mg l⁻¹).

Table 1. Antimicrobial resistance profiles of the A. baumannii isolates used in this study

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Imipenem</th>
<th>Meropenem</th>
<th>Colistin</th>
<th>Polymyxin B</th>
<th>Ampicillin-sulbactam</th>
<th>Tigecycline</th>
<th>Rifampicin</th>
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<tbody>
<tr>
<td>KRU-A-3</td>
<td>128 (R)</td>
<td>&gt;64 (R)</td>
<td>1 (S)</td>
<td>1 (S)</td>
<td>128/64 (R)</td>
<td>2 (S)</td>
<td>2 (S)</td>
</tr>
<tr>
<td>KHU-4</td>
<td>128 (R)</td>
<td>&gt;64 (R)</td>
<td>1 (S)</td>
<td>1 (S)</td>
<td>128/64 (R)</td>
<td>1 (S)</td>
<td>4 (R)</td>
</tr>
<tr>
<td>KCU-4</td>
<td>256 (R)</td>
<td>&gt;64 (R)</td>
<td>32 (R)</td>
<td>4 (R)</td>
<td>256/128 (R)</td>
<td>1 (S)</td>
<td>4 (R)</td>
</tr>
<tr>
<td>SKKU-2</td>
<td>128 (R)</td>
<td>&gt;64 (R)</td>
<td>128 (R)</td>
<td>16 (R)</td>
<td>128/64 (R)</td>
<td>2 (S)</td>
<td>2 (S)</td>
</tr>
<tr>
<td>SKKU-8</td>
<td>128 (R)</td>
<td>&gt;64 (R)</td>
<td>1 (S)</td>
<td>1 (S)</td>
<td>64/32 (R)</td>
<td>16 (R)</td>
<td>2 (S)</td>
</tr>
<tr>
<td>KCU-13</td>
<td>64 (R)</td>
<td>&gt;64 (R)</td>
<td>1 (S)</td>
<td>1 (S)</td>
<td>64/32 (R)</td>
<td>16 (R)</td>
<td>2 (S)</td>
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Escherichia coli ATCC 25922, Staphylococcus aureus ATCC 29213, and Pseudomonas aeruginosa ATCC 27853 were used as control strains.

**Time-kill analysis.** Time-kill studies were performed on five antimicrobial agents (imipenem, colistin, ampicillin-sulbactam, rifampicin and tigecycline) and five combinations of these agents (imipenem plus colistin, imipenem plus ampicillin-sulbactam, colistin plus rifampicin, colistin plus tigecycline, and tigecycline plus rifampicin) according to a previously reported method (Petersen et al., 2006). Time-kill assays were performed in duplicate using concentrations of 0.5× and 1× MIC in both single-agent and combination studies. Bacterial growth was quantified after 0, 2, 4, 8, 12 and 24 h incubation at 37 °C by plating 10-fold dilutions on sheep blood agar. Antimicrobials were considered bactericidal when a ≥ 3 log_{10} decrease in c.f.u. ml⁻¹ was reached compared with the initial inocula. Synergy of the antimicrobial combination was defined as a ≥ 2 log_{10} decrease in c.f.u. ml⁻¹ as compared to use of a single agent (Eliopoulos & Moellering, 1996).

**RESULTS**

**In vitro susceptibilities**

The MICs for imipenem, meropenem, colistin, polymyxin B, ampicillin-sulbactam, tigecycline and rifampicin of the six *A. baumannii* isolates are presented in Table 1. All isolates were resistant to the carbapenems imipenem and meropenem. Additionally, all were resistant to ciprofloxacin, cefepime, ceftazidime, cefoperazone-sulbactam, ceftazidime, piperacillin-tazobactam, tetracycline and ampicillin-sulbactam. While KHU-4 was susceptible to amikacin (MIC 4 mg l⁻¹), the other isolates were resistant (MICs >128 mg l⁻¹).

**Single-agent studies**

Only imipenem was bactericidal against all six *A. baumannii* isolates tested (Table 2); even 0.5× MIC of imipenem resulted in bactericidal effects against two COL-S/TIG-S isolates and one COL-R/TIG-S isolate (KCU-4). Colistin was bactericidal against KCU-4 only (Table 2). Although 1× MIC of colistin initially decreased growth in all *A. baumannii* isolates after 2 and 4 h of incubation, regrowth was observed in five isolates. As observed with the colistin treatment, ampicillin-sulbactam treatment showed a bactericidal effect on KCU-4 and SKKU-8. Even KCU-4 showed about 2 log_{10} regrowth after 24 h of incubation at 1× MIC of ampicillin-sulbactam, compared to that after 12 h of incubation with this agent. None of the *A. baumannii* isolates used in this study were completely killed by tigecycline as a single regimen at either 0.5× or 1× MIC. Rifampicin also had no bactericidal effect against any of the *A. baumannii* isolates tested.

**Combination studies**

Treatment with a combination of 1× MIC imipenem and colistin exerted bactericidal effects on all six *A. baumannii* isolates tested (Table 3, Fig. 1). However, treatment with 0.5× MIC imipenem plus colistin was bactericidal against only four isolates: the two COL-S/TIG-S isolates, a COL-R/TIG-S isolate (KCU-4) and a COL-S/TIG-R isolate (SKKU-8). This combination at 0.5× MIC was not effective against SKKU-2 and KCU-13.

All *A. baumannii* isolates were also not detected in incubations with the combination of 1× MIC imipenem and ampicillin-sulbactam (Table 3). Compared with 1× imipenem alone, three *A. baumannii* isolates (KHU-4, SKKU-2 and KCU-13) were killed earlier by the combination of 1× imipenem and ampicillin-sulbactam. Imipenem plus ampicillin-sulbactam at 0.5× MIC displayed bactericidal activities against all isolates. It is of note that the combination of 0.5× imipenem and ampicillin-sulbactam displayed synergistic and bactericidal effects even against the three isolates (SKKU-2, SKKU-8 and KCU-13) that were not killed within 24 h by 0.5× imipenem alone.

<table>
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<tr>
<th>Table 2. Bactericidal effects of single agents against imipenem-resistant <em>A. baumannii</em> isolates</th>
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<tr>
<td>Resistance*</td>
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<tr>
<td></td>
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<tr>
<td>COL-S/TIG-S</td>
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<td></td>
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<tr>
<td>COL-R/TIG-S</td>
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<tr>
<td></td>
</tr>
<tr>
<td>COL-S/TIG-R</td>
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*COL-S, colistin susceptible; COL-R, colistin resistant; TIG-S, tigecycline susceptible; TIG-R, tigecycline resistant.
†B, bactericidal (when ≥ 3 log_{10} decrease in c.f.u. ml⁻¹ was reached compared with the initial inocula); NB, non-bactericidal.
All tested isolates of bacteria were not detected in incubations with the combination of $1 \times \text{MIC}$ colistin and rifampicin within 8 h after incubation (Table 3). Although KRU-A-3 regrew temporarily after 12 h of incubation, it was eventually eliminated. However, treatment with $0.5 \times \text{MIC}$ colistin plus rifampicin was bactericidal against only two isolates: KCU-4 and KCU-13. Treatment with a combination of $1 \times \text{MIC}$ colistin and tigecycline was also bactericidal against all A. baumannii isolates (Table 3, Fig. 2). Treatment with $0.5 \times \text{MIC}$ colistin plus tigecycline was bactericidal against only two isolates: KCU-4 and KCU-13. Treatment with a combination of $1 \times \text{MIC}$ colistin and tigecycline was also bactericidal or synergistic against only four isolates: one COL-S/TIG-S (KRU-A-3), one COL-R/TIG-S (KCU-4), and the two COL-S/TIG-R isolates. However, SKKU-8 (a COL-S/TIG-R isolate) showed regrowth after 12 h of incubation with $0.5 \times \text{MIC}$ colistin plus tigecycline.

Treatment with the combination of tigecycline and rifampicin was the least effective against the A. baumannii isolates tested (Table 3). Although all isolates reached undetectable levels in incubations with $1 \times \text{MIC}$ tigecycline plus rifampicin, removal took longer (12–24 h) than for the other combinations. The $0.5 \times \text{MIC}$ of tigecycline and rifampicin was synergistic against only two COL-S/TIG-R isolates. Therefore, only two COL-S/TIG-R isolates were not detected by the combination of $0.5 \times \text{MIC}$ tigecycline and rifampicin within 24 h of incubation.

**DISCUSSION**

A. baumannii infections have traditionally been treated with broad-spectrum cephalosporins, $\beta$-lactams and $\beta$-lactamase inhibitors, and carbapenems (Munoz-Price & Weinstein, 2008). However, the emergence of and subsequent increase in MDR A. baumannii isolates, including carbapenem-resistant isolates, have limited the treatment options. Thus, treatment with polymyxins such as polymyxin B and colistin, which had previously been abandoned due to problems of nephrotoxicity and neurotoxicity, is being used for these infections (Peleg et al., 2008; Li et al., 2006b). In addition, tigecycline, a new glycylcycline, has been introduced to treat MDR Gram-negative bacterial infections including A. baumannii (Peleg et al., 2008). However, studies have reported development of resistance to colistin or tigecycline during the treatment (Hawley et al., 2008; Li et al., 2006a; Owen et al., 2007; Peleg et al., 2007). Monotherapy with colistin may be problematic for the treatment of colistin-resistant A. baumannii infections (Owen et al., 2007). Even extreme drug-resistant (XDR) A. baumannii isolates, displaying resistance to all antimicrobials, including polymyxins and tigecycline, have emerged (Doi et al., 2009; Park et al., 2009d). Thus, combination therapy has been recommended not only to combat MDR A. baumannii infections but also to inhibit or reduce the emergence of resistance during treatment.

Not a few studies have been performed on the in vitro activities of combination therapies against A. baumannii infections. Tripodi et al. (2007) reported synergistic effects

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**Table 3. Synergistic effects of antimicrobial combinations against imipenem-resistant A. baumannii isolates**

<table>
<thead>
<tr>
<th>Synergistic effect</th>
<th>Isolate</th>
<th>Imipenem + Ampicillin-sulbactam</th>
<th>Colistin + Tigecycline</th>
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<tbody>
<tr>
<td>0.5x MIC</td>
<td>KRU-A-3</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>1x MIC</td>
<td>KRU-A-3</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>0.5x MIC</td>
<td>KCU-4</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>1x MIC</td>
<td>KCU-4</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>0.5x MIC</td>
<td>KCU-2</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>1x MIC</td>
<td>KCU-2</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>0.5x MIC</td>
<td>SKKU-8</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>1x MIC</td>
<td>SKKU-8</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>0.5x MIC</td>
<td>KCU-13</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>1x MIC</td>
<td>KCU-13</td>
<td>S</td>
<td>S</td>
</tr>
</tbody>
</table>

*COL-S, colistin-susceptible; COL-R, colistin-resistant; TIG-S, tigecycline-susceptible; TIG-R, tigecycline-resistant. NS, non-synergistic.
for combinations of rifampicin plus imipenem or ampicillin-sulbactam against MDR *A. baumannii* isolates. Colistin plus minocycline, tigecycline plus amikacin, carbapenems (imipenem and meropenem) plus polymyxins (polymyxin B and colistin), imipenem plus tigecycline, colistin plus vancomycin, colistin plus teicoplanin, and rifampicin plus sulbactam have also been reported to have synergistic effects (Hornsey & Wareham, 2011; Moland et al., 2008; Pachón-Ibáñez et al., 2006; Gordon et al., 2010; Pankey & Ashcraft, 2009; Sopirala et al., 2010; Tan et al., 2007). In addition, imipenem plus sulbactam and colistin plus rifampicin combinations were effective *in vitro* against carbapenem-resistant *A. baumannii* isolates (Song et al., 2007). However, other *in vitro* time-kill studies demonstrated that tigecycline is ineffective when used in combination with polymyxin B, minocycline, imipenem, levofloxacin, ampicillin-sulbactam and rifampicin against carbapenem-non-susceptible *A. baumannii* isolates (Moland et al., 2008; Scheetz et al., 2007). Overall, many *in vitro* and a few *in vivo* time-kill studies have indicated that antibiotic combination therapy is effective against infections caused by imipenem-resistant *A. baumannii*, although the specific regimens that were shown to be effective differ in each study. To our knowledge, few studies except that of Vila-Farres et al. (2011) have been performed on colistin-resistant *A. baumannii* isolates.

Fig. 1. Effects of imipenem (IMP), colistin (COL), and a combination of these agents on the viability of six imipenem-resistant *A. baumannii* isolates. COL-S, colistin-susceptible; COL-R, colistin-resistant; TIG-S, tigecycline-susceptible; TIG-R, tigecycline-resistant. ◊, 0.5× MIC IMP; ■, 0.5× MIC COL; ◇, 0.5× MIC IMP+COL; ▲, 1× MIC IMP; □, 1× MIC COL; ●, 1× MIC IMP+COL. The *in vitro* time-kill experiments were duplicated; mean values are plotted. In most duplicate experiments, similar time-kill results were obtained.
In contrast to the single-agent experiments, combination regimens displayed excellent bactericidal activities. All imipenem-resistant *A. baumannii* isolates tested were not detected in incubations with all five combinations of antimicrobial agents at 1 × MIC. Although treatment with combinations of antimicrobial agents at 0.5 × MIC was effective against some isolates, the effects were not consistent. Only 0.5 × MIC imipenem plus ampicillin-sulbactam displayed bactericidal activity against all imipenem-resistant *A. baumannii* isolates. As both imipenem and ampicillin-sulbactam target the bacterial cell wall, their combination would be expected to kill the bacteria more rapidly, which may not have clinical implications. Excluding the combination imipenem and ampicillin-sulbactam, 0.5 × MIC colistin plus tigecycline was the most effective, showing synergistic or bactericidal effects against four *A. baumannii* isolates. These results may indicate that increasing the dosage of antimicrobial agents sufficiently is required to achieve a bactericidal effect against resistant *A. baumannii*. However, high dosage of antimicrobial agents may lead to the further emergence of resistance and may increase the toxic effects of those agents. Thus, further investigation of the doses of combination agents that are sufficient to kill bacteria and to prevent the development of resistance is required.

Although the imipenem-resistant *A. baumannii* isolates examined in this study belonged to different PFGE types, they have similar imipenem resistance mechanisms, *bla*<sub>OXA-51</sub> and *bla*<sub>OXA-23</sub>, and all of them belonged to European clone II, an internationally disseminated clone (data not shown). While *bla*<sub>OXA-23</sub> is the main mechanism of imipenem resistance in *A. baumannii*, especially in Korea (Park *et al.*, 2009d; Kim *et al.*, 2010), *A. baumannii* isolates possessing *bla*<sub>IMP</sub> and *bla*<sub>VIM</sub> are also important contributors to imipenem resistance, but such isolates were not included in this study. Our study did not analyse the colistin and tigecycline resistance mechanisms for the *A. baumannii* isolates tested. Thus, our results may not be generalizable to all imipenem-resistant, colistin-resistant or tigecycline-resistant *A. baumannii* isolates. However, our data suggest that some antimicrobial combinations may be effective for combating imipenem-resistant *A. baumannii* infections, including those due to colistin-resistant or tigecycline-resistant bacteria.

**Fig. 2.** Effects of colistin (COL), tigecycline (TIG) and a combination of these agents on the viability of six imipenem-resistant *A. baumannii* isolates. COL-S, colistin-susceptible; COL-R, colistin-resistant; TIG-S, tigecycline-susceptible; TIG-R, tigecycline-resistant. ○, 0.5 × MIC COL; □, 0.5 × MIC TIG; ○, 0.5 × MIC COL+TIG; ●, 1 × MIC COL; ■, 1 × MIC TIG; ●, 1 × MIC COL+TIG. The *in vitro* time-kill experiments were duplicated; mean values are plotted. In most duplicate experiments, similar time-kill results were obtained.
ACKNOWLEDGEMENTS
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