IN VITRO SYNERGISTIC ACTIVITY OF IMIPENEM AND FOSFOMYCIN AGAINST METHICILLIN-RESISTANT STAPHYLOCOCCUS AUREUS

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The in vitro activity of imipenem in combination with fosfomycin against 17 clinical isolates of methicillin-resistant Staphylococcus aureus from hospitalized patients was investigated by checkerboard and time-kill studies. Of the 17 strains tested, checkerboard studies revealed synergy in 14 (82.3%) and indifference in 3 (17.6%): the time-kill studies also revealed synergy in 14 of 14 strains at both 24 and 48 h. The combination of imipenem and fosfomycin may be useful for methicillin-resistant Staphylococcus aureus infections.

Key words: MRSA, Imipenem (IPM), Fosfomycin (FOM), Effect of combination

INTRODUCTION
Infections caused by methicillin-resistant Staphylococcus aureus (MRSA) are becoming increasingly prevalent1,2. At present, vancomycin remains the primary drug used to treat these infections3,4, but its use involves several drawbacks5,6. Imipenem is the first clinically useful member of the carbapenems, a new class of antimicrobial agents that are unique among β-lactams both for their chemical structure and their broad antibacterial spectrum. Since its development from thienamycin as the N-formimidoyl derivative, imipenem has been studied extensively in recent years, and the data available about its in vitro antibacterial activity have been reviewed repeatedly7,8. However, in these studies, investigations of its interaction with other antimicrobial agents are relatively limited. Overall, synergism has been demonstrated in combinations of imipenem with aminoglycosides against enterococci9-12 and Listeria monocytogenes13 and less frequently against staphylococci, enteric Gram-negative bacteria, and nonfermentative bacteria13-17. Conversely, imipenem has shown antagonistic interaction with other β-lactam antimicrobial agents against Pseudomonas aeruginosa18-20 and Serratia marcescens21,22 and with chloramphenicol against Klebsiella pneumoniae21. Fosfomycin is a broad-spectrum bactericidal antibiotic that acts against Gram-positive and -negative bacteria by inhibiting the first step in bacterial cell wall synthesis23. It has been shown to be active against Staphylococcus aureus24,25, including penicillin- and methicillin-resistant strains. It has little or no reported toxicity on serum proteins and does not bind to them23,26.

In this study, MRSA strains isolated from clinical material were tested for susceptibility to imipenem, and fosfomycin. Combinations of imipenem with fosfomycin were then determined both by checkerboard and by time-kill studies.

MATERIALS AND METHODS
MRSA used in this study were isolated from hospitalized patients at Chiba University Hospital. MRSA were decided according to NCCLS27. Namely we decided as MRSA Staphylococcus
** aureus** with MIC of \( \geq 4 \) \( \mu g/ml \) against oxacillin.

Of the 30 strains of MRSA tested, only 17 strains with antibiotic MICs within the range of drug concentrations tested were used in this study. The antibiotics were kindly provided by the manufacturers as reagent-grade powders. Imipenem was obtained from Merck (Japan) Co., Ltd., Tokyo; and fosfomycin was obtained from Meiji Seika Co., Ltd., Tokyo, Japan. Methicillin and oxacillin were obtained from Banyu Co., Ltd., Tokyo, Japan. Potency of imipenem, fosfomycin, methicillin, and oxacillin is 956, 747, 850, and 837 \( \mu g/ml \), respectively. Solutions of each antibiotic were made on the day when they were tested.

Serial twofold dilution of methicillin, oxacillin, fosfomycin, and imipenem were made at 512 to 0.25, 128 to 0.06, 64 to 1, and 32 to 0.03 \( \mu g/ml \) in cation-supplemented Mueller-Hinton broth (CMMHB) (Difco Laboratories, Detroit, Mich.) with 2% NaCl supplementation, respectively, and dispensed into microdilution trays, which were then stored frozen at \(-70^\circ C\).

Broth microdilution testing was performed by methods published by the National Committee for Clinical Laboratory Standards\(^{27}\). The inoculum of the organism was adjusted from a log-phase culture to give a final concentration of \( 5 \times 10^5 \) CFU/ml (\( 5 \times 10^4 \) CFU per well). The covered trays were incubated at \( 35^\circ C \) for 24 h\(^27\). The MIC was defined as the lowest concentration of antibiotic that yielded no visible growth. The MIC\(_{50}\) and MIC\(_{90}\) were the lowest concentrations that inhibited 50 and 90% of the strains.

**In vitro** combinations of imipenem with fosfomycin were assayed by checkerboard studies in 17 strains. The studies were performed by standard microdilution technique in CMMHB with 2% NaCl supplementation. Broth microdilution trays were prepared with an automated system (MIC-2000, Dynatech Laboratories, Inc., Alexandria, Va.) which dispensed 0.1 ml samples of twofold dilutions of imipenem, and fosfomycin in CMMHB with 2% NaCl supplementation. The trays were stored frozen at \(-70^\circ C \) until ready for use. MRSA strains from a log-phase culture in CMMHB with 2% NaCl supplementation at \( 35^\circ C \) were diluted to \( 10^6 \) CFU/ml (MacFarland 0.5) with sterile water. Each well of the checkerboard microdilution broth trays was inoculated with 0.005 ml of the suspension, using a 96-prong automated inoculator (Dynatech Laboratories). Thus, the final inoculum was approximately \( 5 \times 10^5 \) CFU/ml (\( 5 \times 10^4 \) per well). Inoculated trays were covered and incubated for 24 h at \( 35^\circ C \). After 24 h of incubation, the microdilution trays were examined for growth with a mirror reader (Dynatech Laboratories). MICs were recorded as the lowest concentration of antibiotic which suppressed visible growth after 24 h of incubation.

We defined the combination effect by using the fractional inhibitory concentration (FIC) index at the combination point of the lowest concentration of antibiotics suppressing growth after 24 h of incubation at \( 35^\circ C \). We interpreted a combination effect to be synergistic if the FIC index was \( \leq 0.5 \) and antagonistic if the FIC index was \( >4\). Intermediate values were interpreted as indifference.

The time-kill curve method was used to study the combination of imipenem with fosfomycin. Fourteen strains and CMMHB with 2% NaCl supplementation were used. The antibiotic concentrations were as follows: imipenem, 15 \( \mu g/ml \); fosfomycin, 32 \( \mu g/ml \); and the combination of imipenem and fosfomycin, 15 and 32 \( \mu g/ml \), respectively. These are nearly average concentrations obtainable in serum\(^{28,30}\). The inoculum was approximately \( 10^6 \) CFU/ml, diluted from a log-phase culture. All tubes were incubated at \( 35^\circ C \). Samples from each tube were collected at 0, 3, 6, 24, and 48 h of incubation and quantitatively subcultured on blood agar plates. The timed quantitative subculture trays were then incubated at \( 35^\circ C \) for 24 h and the viable number of MRSA was determined for each sampling time. Using the time-kill curve method and combined antibiotics, **in vitro** bactericidal synergy was defined as a minimum of a 100-fold decline in CFU/ml in comparison to the effect of the most active single agent at each sampling time, antagonism as a 100-fold increase in CFU/ml in comparison to the least active single agent, and
indifference as a change in CFU/ml that was neither greater nor less than a 100-fold change in CFU/ml.

RESULTS

As shown in Table 1, the MICs of methicillin for 50% and 90% of the strains tested were 64 and 256 $\mu$g/ml, respectively. The range was 32 to 256 $\mu$g/ml. The MICs of oxacillin for 50% and 90% of the strains tested were 64 and >128 $\mu$g/ml, respectively. The range was 16 to >128 $\mu$g/ml. The MICs of fosfomycin for 50% and 90% of the strains tested were 64 and >128 $\mu$g/ml, respectively. The range was 16 to >128 $\mu$g/ml. The MICs of imipenem for 50% and 90% of the strains tested were 2 and 16 $\mu$g/ml, respectively. The range was 0.5 to 32 $\mu$g/ml. As shown in Table 2, of the 17 strains of MRSA included in the checkerboard study, 14 demonstrated synergy and 3 indifference. No antagonism was observed in this study. Time-kill studies revealed synergy in 9 and 12 of the 14 strains tested at 3 and 6 h, respectively, while the remaining 5 and 2 showed indifference at 3

Table 1. MICs of 17 strains of methicillin-resistant Staphylococcus aureus

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>methicillin</th>
<th>oxacillin</th>
<th>fosfomycin</th>
<th>imipenem</th>
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</thead>
<tbody>
<tr>
<td>34</td>
<td>32</td>
<td>64</td>
<td>64</td>
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<tr>
<td>35</td>
<td>32</td>
<td>16</td>
<td>64</td>
<td>0.5</td>
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<tr>
<td>41</td>
<td>256</td>
<td>&gt;128</td>
<td>64</td>
<td>2</td>
</tr>
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<td>1</td>
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<td>16</td>
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<td>0.5</td>
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<tr>
<td>48</td>
<td>256</td>
<td>&gt;128</td>
<td>64</td>
<td>2</td>
</tr>
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<td>16</td>
<td>64</td>
<td>4</td>
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<td>53</td>
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<td>8</td>
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<td>54</td>
<td>128</td>
<td>16</td>
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<td>61</td>
<td>32</td>
<td>16</td>
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<td>4</td>
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<tr>
<td>62</td>
<td>128</td>
<td>&gt;128</td>
<td>64</td>
<td>16</td>
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</tbody>
</table>

Table 2. Results of checkerboard and time-kill studies of imipenem and fosfomycin against methicillin resistant Staphylococcus aureus (MRSA)

<table>
<thead>
<tr>
<th>Reaction</th>
<th>checkerboard</th>
<th>Time-kill</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3 h</td>
<td>6 h</td>
</tr>
<tr>
<td>Synergy</td>
<td>14</td>
<td>9</td>
</tr>
<tr>
<td>Indifference</td>
<td>3</td>
<td>5</td>
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</table>

The range was 16 to 64 $\mu$g/ml. The MICs of imipenem for 50% and 90% of the strains tested were 2 and 16 $\mu$g/ml, respectively. The range was 0.5 to 32 $\mu$g/ml. As shown in Table 2, of the 17 strains of MRSA included in the checkerboard study, 14 demonstrated synergy and 3 indifference. No antagonism was observed in this study. Time-kill studies revealed synergy in 9 and 12 of the 14 strains tested at 3 and 6 h, respectively, while the remaining 5 and 2 showed indifference at 3
Synergistic activity between IPM and FOM against MRSA

Fig. 1. Time-kill curves of a representative strain (No. 62 strain) of methicillin-resistant Staphylococcus aureus showing synergy between imipenem and fosfomycin

\(\bullet\) : imipenem 15 \(\mu\)g/ml, \(\bigcirc\) : fosfomycin 32 \(\mu\)g/ml, \(\square\) : imipenem 15 \(\mu\)g/ml + fosfomycin 32 \(\mu\)g/ml, \(\bigcirc\) : control

and 6 h, respectively. All 14 strains tested revealed synergy at both 24 and 48 h (Table 2). The time-kill curve of a representative strain (No. 62 strain) of MRSA showing synergy between imipenem and fosfomycin is shown in Fig. 1. The MICs of imipenem and fosfomycin against this strain were 16 and 64 \(\mu\)g/ml, respectively, and its FIC index was 0.37.

**DISCUSSION**

In this study, we investigated the effect of imipenem with fosfomycin against MRSA frequently found in hospitalized patients at the Chiba University Hospital.

Because of its broad spectrum of activity at levels achievable in the blood, imipenem is usually regarded as an antimicrobial agent for use in monotherapy rather than in combination regimens. Hence the practical implications of its *in vitro* interaction with other antimicrobial agents may be inconsequential. Nevertheless, the fact that organisms characteristically tolerant or with a special tendency to develop tolerance to cell-wall-active agents were inhibited and killed synergistically at high rates by particular combinations of imipenem with other antimicrobial agents, at clinically relevant concentrations, is noteworthy and potentially useful. INOUE et al. have reported that the combinations of imipenem with cefazolin, and imipenem with ceftizoxime demonstrated synergistic inhibitory activity against 80% and 96%, respectively, of 25 MRSA strains tested in vitro. In this study, of the 30 strains of MRSA tested, 13 strains were not used because fosfomycin MICs of these strains were outside the range of drug concentrations tested. The combination of imipenem with fosfomycin demonstrated synergistic inhibitory activity against 82.3% of 17 strains of MRSA for which the MIC of fosfomycin was \(\leq\) 64 \(\mu\)g/ml. The time–kill studies also demonstrated that this combination was synergistically active against all 14 strains of MRSA tested. Imipenem alone and fosfomycin alone produced regrowth, but none occurred with the combination, even after 48 h. In our *in vitro* study we demonstrated synergistic activity of this combination against a high percentage of MRSA clinical isolates, but further animal and clinical studies are necessary to confirm these results.

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Synergistic activity between IPM and FOM against MRSA


Methicillin 耐性黄色ブドウ球菌に対する imipenem と fosfomycin との併用効果

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Imipenem (IPM)+fosfomycin (FOM) の併用効果を臨床材料より分離した methicillin 耐性黄色ブドウ球菌（FOM の MIC は ≤64 μg/ml）を用い微量液体希釈法により検討した。相乗作用（FIC index ≤0.5）は、供試菌株 17 株中 14 株 82.3％に、不関連 3 株 17.6％に認められた。また IPM+FOM の組み合わせは、殺菌曲線でも 24 と 48 時間目に 14 株中 14 株に相乗効果が認められ、methicillin 耐性黄色ブドウ球菌感染症に対する臨床的有用性がうかがわれた。

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