In Vitro Effects of β-Lactams Combined with β-Lactamase Inhibitors against Methicillin-Resistant *Staphylococcus aureus*

SHINZO KOBAYASHI, SUSUMU ARAI, SHORYO HAYASHI,* AND TAKASHI SAKAGUCHI

*Pharma Research Laboratories, Hoechst Japan Limited, 1-3-2 Minamidai, Kawagoe, Saitama 350, Japan*

Received 21 April 1988/Accepted 16 December 1988

The effects of combinations of β-lactams with two β-lactamase inhibitors, sulbactam and clavulanic acid, were determined in vitro against 22 clinical isolates of methicillin-resistant *Staphylococcus aureus*. Combinations of cefpirome, cefotaxime, and cefazolin with sulbactam (10 μg/ml) showed synergistic effects against more than 70% of the strains. Combinations of methicillin and penicillin G with sulbactam also showed synergistic effects against 50 and 68% of the strains, respectively, while cefotiam, moxalactam, flomoxef, and cefmetazole in combination with sulbactam showed such effects against only 40% or fewer. Clavulanic acid was synergistic only when combined with penicillin G, the effect probably being due to the β-lactamase inhibition by the inhibitor. Sulbactam did not improve the antimicrobial activities of the β-lactams against methicillin-susceptible *S. aureus* strains. At 42°C the MICs of cefotaxime, methicillin, and flomoxef alone were markedly decreased from the values at 35°C, and no synergy between these β-lactams and sulbactam appeared. The resistance to penicillin G was not inhibited by incubation at 42°C, and combinations of penicillin G with sulbactam and clavulanic acid showed synergy. The amounts of β-lactamase produced were not related to the decreases in the MICs of the β-lactams, except for penicillin G combined with sulbactam. Clavulanic acid showed slightly stronger β-lactamase-inhibiting activity than sulbactam did. These results suggest that the synergy between sulbactam and the β-lactams, except for penicillin G, may not be due to β-lactamase inhibition but to suppression of the methicillin-resistant *S. aureus*-specific resistance based on other factors.

Recently, reports have been made on the isolation of and infections with methicillin-resistant *Staphylococcus aureus* (MRSA) (1, 5, 9, 25, 26). The resistance of these strains is associated with the presence of a low-affinity penicillin-binding protein, PBP 2' (23) or PBP 2a (10), and the protein has been suggested to be inducible by β-lactams (7, 18, 22). The role of β-lactamase in the resistance has also been examined with the β-lactamase inhibitors clavulanic acid (17, 18) and sulbactam (3, 8), and it has been reported that staphylococcal β-lactamase may be the cause of decreased antimicrobial activities of drugs such as penicillin G and cefazolin, which are relatively susceptible to β-lactamase hydrolysis (4, 15). There has also been a report that drug inactivation by penicillinase is the main possible mechanism of resistance to cefazolin, cephaloridine, and cephalothin in *S. aureus* (14).

To determine the role of β-lactamase in the resistance to more antimicrobial agents, we determined the effects of combinations of nine β-lactams with the two β-lactamase inhibitors in vitro against clinically isolated MRSA strains. The β-lactams examined were cefpirome (20) and flomoxef (21), which have strong antimicrobial activities against *S. aureus* as well as gram-negative bacteria; five other cephalosporins (cefotaxime, moxalactam, cefmetazole, cefotiam, and cefazolin); and two penicillins (penicillin G and methicillin). Interestingly, we found that the activities of some β-lactamase-stable cephalosporins and methicillin were markedly improved when these drugs were combined with sulbactam but that no such synergy occurred when they were combined with clavulanic acid. In addition, the inhibitory effects of the two β-lactamase inhibitors on the β-lactamases from four MRSA strains were also examined.

**MATERIALS AND METHODS**

**Bacterial strains.** Twenty-two MRSA strains were used; they were clinically isolated over the past few years and provided by the Department of Bacteriology, Faculty of Medicine, Iwate Medical University (Iwate, Japan). For all strains the methicillin MICs were 12.5 μg/ml or more when determined by the agar dilution method. Methicillin-susceptible, penicillinase-negative *S. aureus* strains were FDA 209P JC-1, IAM 1011, and ATCC 25923, which were maintained in our laboratory. All bacterial strains were suspended in 0.1 M phosphate buffer (pH 7.0) containing 25% glycerin and stored at −70°C until use.

**Antimicrobial agents.** Cefpirome and pyridine-2-azo-p-dimethylaniline cephalosporin (PADAC) (12) were supplied by Hoechst AG (Frankfurt, Federal Republic of Germany). The other agents were obtained as follows: cefotaxime (Hoechst Japan Ltd., Tokyo, Japan), cefazolin and carbencillin (Fujisawa Pharmaceutical Co. Ltd., Osaka, Japan), flomoxef and moxalactam (Shionogi & Co., Ltd., Osaka, Japan), cefmetazole (Sankyo Co., Ltd., Tokyo, Japan), cefotiam (Takeda Chemical Industries, Ltd., Osaka, Japan), methicillin (Banyu Pharmaceutical Co., Ltd., Tokyo, Japan), cephaloridine (Torii and Co., Ltd., Tokyo, Japan), cloxacillin (Meiji Seika Kaisha Ltd., Tokyo, Japan), penicillin G and sulbactam (Pfizer Taito Co., Ltd., Tokyo, Japan), and clavulanic acid (Beecham Yakuin K.K., Tokyo, Japan).

**MIC determinations.** The MICs were determined by the agar dilution method. Twofold serial dilutions of each β-lactam alone or in combination with sulbactam or clavulanic acid were prepared in sensitivity test agar (modified Mueller-Hinton agar; Nissui Seiyaku Co., Ltd., Tokyo, Japan). The concentrations of the β-lactamase inhibitors were 1 and 10 μg/ml, less than one-fourth the respective MICs for each bacterial strain (10 μg of clavulanic acid per ml for methicillin-susceptible *S. aureus* was close to the MIC, so this...
concentration was not tested for its combination effects). A fresh overnight culture of each strain in sensitivity test broth (modified Mueller-Hinton broth; Nissui) was diluted with the same broth, and inocula of about 10^5 CFU per spot were applied onto drug-containing agar media with an inoculum-replicating apparatus (Microplanter; Sakura, Tokyo, Japan). The MIC was read as the lowest concentration of each β-lactam that inhibited the development of visible bacterial growth on the agar after 24 h of incubation at 35 or 42°C. Combination effects were defined, as follows, as compared with the MIC of each β-lactam alone: synergy, fourfold or greater decrease in the MIC; and antagonism, fourfold or greater increase in the MIC.

**β-Lactamase preparation.** β-Lactamase was isolated from all 22 MRSA strains as follows. Each strain was cultured first in 10 ml of sensitivity test broth at 35°C overnight and then in 100 ml of fresh sensitivity test broth either containing or not containing 2 μg of an inducer (methylisicillin) per ml with shaking at 35°C for 6 h. The culture was centrifuged at 7,200 × g for 15 min at 4°C, and harvested cells were washed twice with 50 mM phosphate buffer (pH 7.0) and suspended in 3 ml of the same buffer. Lysostaphin (Sigma Chemical Co., St. Louis, Mo.) was added to the suspension to yield a final concentration of 100 μg/ml, and the mixture was incubated at 35°C for 30 min. Cells were disrupted by ultrasonic treatment (40 W, 15 min, 80% duty cycle) with a sonicator (model 375; Heat Systems-Ultrasonics Inc., New York, N.Y.) in an ice bath, and cell debris was removed by centrifugation at 16,000 × g for 15 min at 4°C. The supernatant obtained was used as a crude enzyme and stored at −70°C until use.

**β-Lactamase assay.** The β-lactamase assay was performed at 35°C with penicillin G, carbenicillin, cloxacillin, cephaloridine, and PADAC as substrates by the method described previously (13). One unit of enzyme activity was defined as the amount of enzyme hydrolyzing 1 μmol of each substrate in 1 min. The protein concentration was determined by the method of Waddell (24). The substrate profile of each strain was examined by determination of the relative rates of hydrolysis of the substrates. The enzyme inducibility of each strain was determined with penicillin G as a substrate. The inhibition of β-lactamase by sulbactam and clavulanic acid was examined as follows. To the crude enzyme obtained each inhibitor was added at a final concentration of 10 μg/ml, and the mixture was incubated at 35°C for 15 or 90 min. The enzyme activity in the incubated enzyme-inhibitor mixture was determined as described above with penicillin G as a substrate.

---

**RESULTS**

**Combination effects against MRSA strains.** Table 1 shows the median MICs of the β-lactams in combination with sulbactam and clavulanic acid determined at 35°C. The two β-lactamase inhibitors used alone showed only weak antimicrobial activities, and their concentrations used in combinations were less than one-fourth the respective MICs.

Sulbactam combined with cefpirome, cefotaxime, cefazolin, methicillin, and penicillin G improved their antimicrobial activities. The synergistic effects of sulbactam were marked, especially in combination with cefpirome and cefotaxime, and the median MICs of these combinations were almost comparable to that of flomoxef, which had the lowest MIC among the β-lactams used alone. Interestingly, the MIC ranges of these five β-lactams in combination with the β-lactamase inhibitor remained unchanged from the MIC ranges of the drugs alone. This result suggests that the population of MRSA used probably included heterogeneous strains. When the median MICs of each β-lactam alone were examined separately for the strains for which synergy was observed when the drug was combined with sulbactam (10 μg/ml) and for the strains for which synergy was not observed, there was no difference (no change or only a twofold change) in the median MICs of the β-lactams, except for flomoxef. For flomoxef, a great difference (16-fold increase in the MIC) was noted between the former and latter strains, suggesting that the combination of flomoxef with sulbactam was synergistic against the MRSA strains with high MICs. Among the β-lactams combined with clavulanic acid, only penicillin G showed synergy.

Table 2 shows the number of strains for which synergy (fourfold or greater decrease in the MIC) was observed with sulbactam or clavulanic acid. Sulbactam (1 μg/ml) in combinations with cefpirome and cefotaxime showed synergy against 50% or more of the strains. With 10 μg of sulbactam per ml synergy occurred against larger numbers of strains. The cefpirome, cefotaxime, and cefazolin combinations showed synergy against more than 70% of the strains, and the methicillin and penicillin G combinations were also synergistic against 50% or more. However, no synergy appeared with moxalactam or ceftiom. Synergy was observed for 27% of strains with flomoxef. In combination with clavulanic acid, only penicillin G showed synergy; with 10 μg of this inhibitor per ml synergy was noted against 59% of the strains.

In the presence of sulbactam (10 μg/ml), there were many strains for which synergy was observed with multiple β-


lactams. Sulbactam plus ceftipime, cefotaxime, cefazolin, methicillin, or penicillin G showed synergy against the same seven strains. Sulbactam plus cefazolin or cefotaxime was synergistic against the same 17 strains, and included among them were 15, 9, and 11 strains for which synergy was noted with sulbactam plus ceftipime, methicillin, or penicillin G, respectively (equivalent to 94, 81, and 75% of the strains for which synergy appeared with the respective compounds). In addition, five strains for which sulbactam plus cefmetazole or flomoxef showed synergy were also included in the strains for which synergy was observed with sulbactam plus ceftipime, cefotaxime, cefazolin, or methicillin.

Combination effects against methicillin-susceptible *S. aureus* and resistance-depressed MRSA strains. To examine whether the synergy between the β-lactams and sulbactam was specific for the resistance in MRSA strains, we determined the MICs of β-lactams combined with sulbactam (10 μg/ml) against three methicillin-susceptible *S. aureus* strains and against the 22 MRSA strains at 42°C, at which their resistance was reported to be reduced (2).

The MICs of the β-lactams and β-lactamase inhibitors for the three methicillin-susceptible strains were as follows (in micrograms per milliliter): ceftipime, 0.39; cefotaxime, 1.56 to 3.13; moxalactam, 6.25 to 12.5; flomoxef, 0.39; cefmetazole, 0.78 to 3.13; cefotiam, 1.56; cefazolin, 0.2; methicillin, 1.56; penicillin G, 0.025; sulbactam, 100 to 200; and clavulanic acid, 12.5 to 25. The addition of sulbactam at 10 μg/ml either did not change the MIC or caused only a twofold decrease in the MIC of any β-lactam. Thus, 10 μg of sulbactam per ml had no synergistic effect on the activities of the β-lactams. Sulbactam alone, like the other β-lactams alone, had much lower MICs for these susceptible strains than for the MRSA strains. No antagonism occurred between any β-lactam and sulbactam.

The MICs of cefotaxime, flomoxef, methicillin, and penicillin G alone and in combination with 10 μg of sulbactam or clavulanic acid per ml were determined against the 22 clinical isolates of MRSA after 24 h of incubation at 42°C (Table 3). At 42°C, the median MICs of cefotaxime, methicillin, and flomoxef alone decreased 32-, 32-, and 8-fold, respectively, from those at 35°C, and the expression of bacterial resistance to these drugs was inhibited. By contrast, the MIC of penicillin G showed only a twofold decrease, and the resistance to this drug remained unchanged after incubation at 42°C. The median MICs of cefotaxime, methicillin, and flomoxef combined with the β-lactamase inhibitors remained unchanged or showed only a twofold decrease from the median MICs of the drugs alone (at 42°C); thus, no synergy occurred. For combinations of sulbactam with cefotaxime and methicillin, this result was in contrast with the result at 35°C that the MICs of these combinations decreased eight- or fourfold from the MICs of the drugs alone (Table 1). On the other hand, penicillin G combined with sulbactam or clavulanic acid showed synergy at 42°C against 73 and 91% of the strains, respectively; the respective values at 35°C were 68 and 59%.

**β-Lactamase activity and inducibility.** All 22 MRSA strains produced β-lactamase inducibly when methicillin was used as the inducer, although the enzyme activity varied among the strains. The mean β-lactamase activities plus or minus the standard errors for the 22 strains were 0.10 ± 0.02 (range, 0.003 to 0.29) U/mg of total protein in the absence of the inducer and 2.45 ± 0.49 (range, 0.02 to 7.41) U/mg of total protein in the presence of the inducer. The ratio of the induced enzyme activity to the noninduced enzyme activity was 27.2 ± 4.9 (1.7 to 91.5) and varied widely among the strains.

When the rate of hydrolysis of penicillin G by the enzyme from each strain was taken as 100%, the relative rates of carbencillin hydrolysis by the enzymes from the 22 strains were 20 to 30%, and the values for cloxacillin, cephaloridine, and PADAC were all less than 1%. Little difference appeared among the strains. From this substrate profile, we concluded that all MRSA strains produced the same type of enzyme (penicillinase).

The relationship between the synergistic effects of sulbactam (10 μg/ml) and β-lactamase activity was examined. The enzyme activity varied widely among the strains, and no significant difference was noted between the strains for which synergy was observed and the strains for which synergy was not observed with any combination of β-lactams (0.6 to 2.0 ratio of the former to the latter strain values), except for penicillin G (2.8). As for enzyme inducibility (ratio of induced enzyme activity to noninduced enzyme activity), there was no significant difference between the strains for which synergy appeared and the strains for which synergy did not appear with any β-lactam combination (0.6 to 1.4 ratio of the former to the latter strain values). A statistical comparison was made by Student's *t* test, and no observed difference had a value of *P* < 0.05.

**Inhibition of β-lactamase by sulbactam and clavulanic acid.** Table 4 shows the activities of methicillin-induced β-lactamases from four MRSA strains and the inhibitory effects of the two β-lactamase inhibitors (10 μg/ml) on them. Both inhibitors, when incubated with β-lactamase for 15 min, strongly inhibited the enzyme activity. Their inhibitory effects tended to be dependent on the incubation period. Clavulanic acid showed somewhat stronger effects than sulbactam. Of the four strains, HL1185 and HL1249 were those against which ceftipime, cefotaxime, cefazolin, methicillin, and penicillin G in combination with sulbactam (10 μg/ml) showed synergy. The inhibitory effects of sulbactam on these strains

### Table 2. Incidence of synergy between β-lactams and β-lactamase inhibitors in 22 MRSA strains

<table>
<thead>
<tr>
<th>β-Lactam</th>
<th>Sulbactam at:</th>
<th>Clavulanic acid at:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 μg/ml</td>
<td>10 μg/ml</td>
</tr>
<tr>
<td></td>
<td>1 μg/ml</td>
<td>10 μg/ml</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>13 (59)</td>
<td>16 (73)</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>11 (50)</td>
<td>17 (77)</td>
</tr>
<tr>
<td>Cefepime</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Cefepime</td>
<td>6 (27)</td>
<td>26 (72)</td>
</tr>
<tr>
<td>Cefmetazole</td>
<td>2 (9)</td>
<td>5 (23)</td>
</tr>
<tr>
<td>Cefotiam</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Cefazolin</td>
<td>1 (5)</td>
<td>17 (77)</td>
</tr>
<tr>
<td>Methicillin</td>
<td>6 (27)</td>
<td>11 (50)</td>
</tr>
<tr>
<td>Penicillin</td>
<td>4 (18)</td>
<td>15 (68)</td>
</tr>
</tbody>
</table>

### Table 3. Effects of combinations of β-lactams with β-lactamase inhibitors against 22 MRSA strains at 42°C

<table>
<thead>
<tr>
<th>β-Lactam</th>
<th>Median MIC (range) (μg/ml) of drug:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Alone</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>6.25 (3.13–25)</td>
</tr>
<tr>
<td>Flomoxef</td>
<td>0.78 (0.39–3.13)</td>
</tr>
<tr>
<td>Methicillin</td>
<td>3.13 (1.56–12.5)</td>
</tr>
<tr>
<td>Penicillin G</td>
<td>25 (1.56–50)</td>
</tr>
</tbody>
</table>

Thus, no synergy occurred. For combinations of sulbactam with cefotaxime and methicillin, this result was in contrast with the result at 35°C that the MICs of these combinations decreased eight- or fourfold from the MICs of the drugs alone (Table 1). On the other hand, penicillin G combined with sulbactam or clavulanic acid showed synergy at 42°C against 73 and 91% of the strains, respectively; the respective values at 35°C were 68 and 59%.
were not much different from those on the two other strains, HL1195 and HL1260, against which no synergy was noted.

**DISCUSSION**

The role of β-lactamase in the resistance of MRSA strains to β-lactams has already been studied by other investigators. Boyce and Medeiros found that clavulanic acid inhibited the development of bacterial resistance to penicillin G and cefazolin, which are relatively susceptible to staphylococcal β-lactamase hydrolysis, but that it did not affect the activities of β-lactamase-stable drugs such as methicillin, oxacillin, and cephalothin (4). McDougall and Thornsberry reported that the addition of sulbactam and clavulanic acid increased the susceptibility of MRSA strains to penicillin G but did not affect their susceptibility to methicillin, oxacillin, or cephalothin (15). Our results with clavulanic acid combined with methicillin or penicillin G were in agreement with their results. Unlike clavulanic acid, however, sulbactam showed synergistic effects when it was used along with methicillin and some other β-lactams, including cefpirome and cefotaxime. These were unexpected results, and the results with methicillin were contrary to those reported by McDougall and Thornsberry. In an attempt to clarify the cause of this difference, we also tested the sulbactam-β-lactam combinations on 2% NaCl agar medium, as described in their report (15), but we again obtained the same results, i.e., synergy (data not shown). Therefore, the cause may be associated with other factors, such as differences in test strains, media, and MIC determination methods.

In the present study, we did not examine the effects of combinations on β-lactamase-nonproducing MRSA strains, and the possibility cannot be denied that the β-lactams may have been inactivated at a very low rate of hydrolysis. However, the following results suggest that the synergy noted between sulbactam and the β-lactams may not have occurred because of the inhibition of staphylococcal β-lactamase by sulbactam. (i) Clavulanic acid, which had slightly greater β-lactamase-inhibiting effects than sulbactam did and strongly inhibited the enzymes isolated from the MRSA strains, did not augment the antimicrobial activities of the β-lactams, except for penicillin G. (ii) There was no significant difference in enzyme activity or inducibility between the strains for which synergy was observed and those for which synergy was not observed with any combination of β-lactams, except for penicillin G. Penicillin G has a relatively low enzyme stability and, when combined with sulbactam, seemed to be more synergistic against strains with higher enzyme activities. (iii) Sulbactam was more likely to show synergy in combination with the cephalosporins than in combination with penicillin G. The substrate profile indicated that the MRSA strains produced penicillinase; therefore, if sulbactam is effective in inhibiting the enzyme, in combination with penicillin G it could have shown synergy more frequently. (iv) Synergy did not occur between sulbactam and the β-lactams, except for penicillin G, at 42°C, at which the strains were resistant only to penicillin G. Combinations of penicillin G with the two β-lactamase inhibitors more frequently showed synergy at 42 than at 35°C. This result suggests that both β-lactamase and a temperature-sensitive, MRSA-specific factor (e.g., an altered penicillin-binding protein) may be associated with bacterial resistance to penicillin G and that only the latter factor may be associated with resistance to other β-lactamase-stable β-lactams such as methicillin.

The mechanism of resistance of MRSA strains to β-lactams has been examined in several studies. It has been found that the resistance is due to the production of a penicillin-binding protein having a low affinity for the β-lactams, PBP 2' (22, 23) or PBP 2a (10), and that this penicillin-binding protein is inducible by the β-lactams themselves (7, 16, 19, 22). From these findings, the following explanation is proposed to account for the different synergistic effects caused by the two β-lactamase inhibitors, sulbactam and clavulanic acid, in combination with β-lactams: (i) sulbactam has a lower PBP 2'-inducing activity than clavulanic acid does and suppresses the induction of PBP 2' by other β-lactams, or (ii) sulbactam has a higher affinity for PBP 2' than clavulanic acid does and inhibits the expression of PBP 2'-induced resistance in MRSA strains.

Recently, Hartman and Tomasz referred to the possibility that an undefined factor (factor X) other than PBP 2a or PBP 2' controls the expression of methicillin resistance (11). Murakami et al. found that the amount of PBP 2' induced in MRSA strains by flomoxef did not correlate with its MIC, suggesting that another unknown factor contributes to the increased MIC (16). Chambers and Hackbarth also suggested that an additional factor besides PBP 2a which may act within the autolytic pathway is required for the expression of resistance in MRSA strains (6). Therefore, it cannot be denied that the synergy observed between sulbactam and the β-lactams may be produced by effects of sulbactam on such an unknown factor involved in resistance in MRSA strains.

When combined with sulbactam, cepfriome and cefotaxime, which have a methoxy-imino group, showed high incidences of synergy, whereas moxalactam, cefmetazole, and flomoxef, which have a methoxy group at the 7 position, had low incidences. A study into the cause of this difference from a structural point of view may also be helpful in clarifying the mechanism of the synergy.

In the present study, sulbactam and clavulanic acid, usually classified as β-lactamase inhibitors, were found to have properties other than β-lactamase inhibition. These differences should be noted when the role of β-lactamase in resistance in MRSA and other β-lactam-resistant bacteria is studied with these β-lactamase inhibitors.

**LITERATURE CITED**


---

**TABLE 4. Inhibition by sulbactam and clavulanic acid (10 µg/ml) of β-lactamases derived from four MRSA strains**

<table>
<thead>
<tr>
<th>Strain</th>
<th>β-Lactamase activity* (U/mg of protein)</th>
<th>Remaining β-lactamase activitya (%) after treatment for the indicated time with sulbactam</th>
<th>Clavulanic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>15 min</td>
<td>90 min</td>
</tr>
<tr>
<td>HL1185</td>
<td>2.48</td>
<td>10.4</td>
<td>1.6</td>
</tr>
<tr>
<td>HL1195</td>
<td>0.13</td>
<td>7.9</td>
<td>4.8</td>
</tr>
<tr>
<td>HL1249</td>
<td>3.17</td>
<td>3.1</td>
<td>2.9</td>
</tr>
<tr>
<td>HL1260</td>
<td>3.58</td>
<td>4.4</td>
<td>3.3</td>
</tr>
</tbody>
</table>

* Induced by methicillin (2 µg/ml).

* β-Lactamase activity without an inducer was taken as 100%.
compound which acts as a β-lactamase inhibitor. J. Antibiot. 31:1238–1244.


