The in vivo activity of BO-3482, which has a dithiocarbamate chain at the C-2 position of 1β-methyl-carbapenem, was compared with those of vancomycin and imipenem in murine models of sepsisemia and thigh infection with methicillin-resistant \textit{Staphylococcus aureus} (MRSA). Because BO-3482 was more susceptible than imipenem to renal dehydropeptidase I in a kinetic study of hydrolysis by this renal enzyme, the therapeutic efficacy of BO-3482 was determined during coadministration with cilastatin. In the septicemia models, which involved two homogeneous MRSA strains and one heterogeneous MRSA strain, the 50% effective doses were, respectively, 4.80, 6.06, and 0.46 mg/kg of body weight for BO-3482; 5.56, 2.15, and 1.79 mg/kg for vancomycin; and >200, >200, and 15.9 mg/kg for imipenem. BO-3482 was also as effective as vancomycin in an MRSA septicemia model with mice with cyclophosphamide-induced immunosuppression. In the thigh infection model with a homogeneous MRSA strain, the bacterial counts in tissues treated with BO-3482-cilastatin were significantly reduced in a dose-dependent manner compared with the counts in those treated with vancomycin and imipenem-cilastatin ($P < 0.001$). These results indicate that BO-3482–cilastatin is as effective as vancomycin in murine systemic infections and is more bactericidal than vancomycin in local-tissue infections. The potent in vivo activity of BO-3482–cilastatin against such MRSA infections can be ascribed to the good in vitro anti-MRSA activity and improved pharmacokinetics in mice when BO-3482 is combined with cilastatin and to the bactericidal nature of the carbapenem.

Although more than 30 years have passed since the first report of methicillin-resistant \textit{Staphylococcus aureus} (MRSA) (13), MRSA still presents a serious problem as a cause of nosocomial infections worldwide (19). Vancomycin, a cyclic glycopeptide antibiotic, is extensively used in clinics to treat MRSA infections; however, this antibiotic is not an ideal anti-biotic because of the slow clinical response to this agent (12) and its possible adverse effects (4). In the late 1980s, methicillin-resistant coagulase-negative staphylococci (20) and vancomycin-resistant enterococci began to appear (9, 24). The possibility that vancomycin-resistant MRSA strains are emerging is suggested by the demonstration that vancomycin resistance genes can transfer from enterococci to \textit{S. aureus} and be expressed by \textit{S. aureus} (16). Therefore, it is necessary to develop a new antibiotic that is clinically useful against MRSA.

Recently, various anti-MRSA β-lactams such as the carbapenems L-695,256 (3) and SM-17466 (21) and the cephems TOC-39 (6), FK-037 (23), and 2-oxacephems (22) have been described.

In our laboratory, a new 1β-methyl-carbapenem, BO-3482 sodium, (1R,5S,6S)-6-[(R)-1-hydroxyethyl]-2-[N-(2-hydroxyethyl)-N-methyl amino] thiocarbonylthio]-1-methyl-1-carbapen-2-εm-3-carboxylate (Fig. 1), was discovered in the course of derivatization directed toward anti-MRSA carbapenems (1, 7). The introduction of a dithiocarbamate side chain at the C-2 position of 1β-methyl-carbapenem led to good binding to PBP 2’ (or PBP 2a) of MRSA, which reflected high activity against homogeneous MRSA. In this report, we describe the in vivo antimicrobial activity of BO-3482 against MRSA strains and compare it with those of vancomycin and imipenem in murine sepsisemia and thigh infection models.

**MATERIALS AND METHODS**

**Antibiotics.** BO-3482 was synthesized at the Tsukuba Research Institute, Banyu Pharmaceutical Co., Ltd., Tsukuba, Japan. Imipenem and cilastatin sodium were the products of Banyu Pharmaceutical Co., Ltd. Vancomycin was purchased from Sigma Chemical Co., St. Louis, Mo. The solutions of antimicrobial agents were freshly prepared on the day of use. In the in vitro study, vancomycin was dissolved in 50 mM phosphate buffer (pH 7.0) and BO-3482 and imipenem were dissolved in 50 mM 3-(N-morpholino)propanesulfonic acid (MOPS) buffer (pH 7.0). In the pharmacokinetic and therapeutic efficacy studies, the antibiotics were dissolved in saline or cilastatin-saline. Cilastatin was coadministered at a dose of 40 mg per kg of body weight per dose in treatments with vancomycin or BO-3482.

**Bacterial strains.** Clinical isolates of MRSA, isolates BB6221, BB6226, and BB6156, which were collected from several hospitals in Japan over the past several years, and methicillin-susceptible \textit{S. aureus} (MSSA) Smith were used for the murine sepsisemia model. MRSA BB6224 was used for the murine thigh infection model. MRSA BB6221, BB6226, and BB6294 are β-lactamase negative and homogeneously resistant, while strain BB1658 is β-lactamase positive, inducible, and heterogeneously resistant. β-Lactamase production was investigated by incubating the cell suspension with nitrocefin, a chromogenic cephalosporin (17). The inducibility was determined by the same method in the absence or presence of a sub-MIC of imipenem, an inducer. All bacteria were maintained in glycerol broth at −80°C.

**Susceptibility tests.** MICs were determined by the twofold agar dilution method with Mueller-Hinton medium (Difco Laboratories, Detroit, Mich.). The medium was supplemented with 2% NaCl for MRSA. An overnight culture grown at 37°C in Mueller-Hinton broth (Difco) was diluted to $10^6$ CFU/ml and was inoculated onto a drug-containing agar surface with an inoculum apparatus (Microplanter; Sakuma Seisakusyo, Tokyo, Japan). The final inoculum size was approximately $10^6$ cells per spot. The MIC was defined as the lowest concentration that inhibited visible growth after 18 h of incubation at 37°C for MSSA and 48 h of incubation at 35°C for MRSA.

**Stability against renal DHP-I.** The stabilities of the carbapenems against renal dehydropeptidase I (DHP-I) were determined with partially purified murine and porcine renal DHP-I. Enzyme was prepared by the procedure described previously (2). The activity of DHP-I was spectrophotometrically determined by...

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measuring the hydrolysis of glycyldedehydophenylalanine as a substrate. One unit of enzyme activity was defined as the amount of enzyme that hydrolyzed 1 μmol of substrate per minute per mg of protein at 35°C. The rate of enzyme-catalyzed hydrolysis of carbapenems was measured spectrophotometrically, and reactions were carried out in 50 mM MOPS buffer (pH 7.0) at 35°C. The respective millimolar extinction coefficients per centimeter (ε0) were as follows: BO-3482, 12.83 at 300 nm; imipenem, 9.04 at 299 nm. The Michaelis constant (Km) and maximum rate (Vmax) of the hydrolytic reactions of the enzymes were determined from a Lineweaver-Burk plot of the initial velocity of carbapenem hydrolysis by renal DHP-I.

Mice. ICR mice (age, 4 weeks) were obtained from Charles River Japan Inc., Yokohama, Japan. When necessary, the mice were immunosuppressed by injecting 250 mg of cyclophosphamide (Shionogi Pharmaceutical Co., Ltd., Osaka, Japan) per kg of body weight intraperitoneally 4 days before infection in a manner similar to that described previously (18). This procedure produced severe neutropenia (<100 neutrophils per mm³) on the day of the experiment.

Infection models. The in vivo activities of the drugs were determined with mice with S. aureus systemic or thigh infections. (i) Systemic infection. In the septicemia model, seven or eight mice were used for each dose of drug. Late-exponential-phase S. aureus cells on brain heart infusion agar (Difco) were harvested and suspended in 5% gastric mucin (Difco). A 0.5-ml portion of the bacterial suspension, corresponding to a dose at least three times higher than the 50% lethal dose (LD50) was inoculated intraperitoneally into each mouse. Under these conditions, all untreated mice died within 3 days. In therapeutic efficacy studies, at least five doses of antibiotic (serial 2- to 3.5-fold dilutions were used) were administered subcutaneously, taking 200 μg of the antibiotic per kg as the highest dose. Normal mice received a single dose of each antibiotic 1 h after injection, while immunosuppressed mice received two doses of drug 1 and 3 h after infection. The number of mice surviving at each dose was counted 6 days after infection, and the 50% effective doses (ED50s) were calculated by the probit method (14).

(ii) Thigh infection. In the thigh infection model, four immunosuppressed mice were used per group. An overnight culture of MRSA BB6269 in tryptic soy broth (Difco) was washed and resuspended in fresh tryptic soy broth to ca. 10⁸ CFU/ml. Of this suspension, 0.1 ml was injected into the right thighs of slightly anesthetized mice. The mice received two doses of drug subcutaneously 2 and 6 h after infection of the test organisms by the following treatment regimens: 10, 20, or 40 mg of BO-3482 with 40 mg of cilastatin per kg per dose; 10, 20, or 40 mg of vancomycin per kg per dose; or 40 mg of imipenem with 40 mg of cilastatin per kg per dose. The dosages were chosen on the basis of the possible maximum dose projected for therapy in humans. Four hours after the last treatment, the thigh muscles were removed and immediately homogenized in ice-cold 0.9% NaCl with a tissue homogenizer (Ystral, Gottingen, Germany). Viable cells counts were determined on Mueller-Hinton medium by plating duplicate samples of the homogenate onto paper disks (8 mm in diameter), and the disks were then placed on the inoculated agar plates in triplicate. The plates were incubated at 37°C overnight, and the zones of inhibition were measured. The potencies of the test samples were calculated from the standard curve.

Pharmacokinetic parameters were calculated by the moment method (25). The maximum concentration of drug in plasma (Cmax) was directly determined from the profiles of the concentration in plasma. The elimination rate constant (k12) was calculated from linear regression analysis of the plasma concentration-time curve. The half-life (t1/2) was calculated as ln2/k1. The area under the plasma concentration-time curve (AUC) was determined by using the trapezoidal rule and was extrapolated to infinity.

### RESULTS

**Stability to renal DHP-I.** The kinetic parameters for murine and porcine renal DHP-I in the hydrolysis of BO-3482 and imipenem are presented in Table 1. The Kmo and Vmax values of DHP-I for BO-3482 varied with the source of the enzyme. In both cases, BO-3482 showed a higher affinity for DHP-I than imipenem did, since the Kmo values of murine and porcine DHP-I for BO-3482 were 1/4 and 1/32 of those for imipenem, respectively. The relative Vmax of porcine DHP-I for BO-3482 when the hydrolysis rate of imipenem was taken as 100 was about one-eighth that of murine DHP-I. The relative Vmax/Km values of murine and porcine DHP-I were 1.74 and 1.65, respectively, with those for imipenem being taken as 1. Thus, BO-3482 was more readily hydrolyzed by murine and porcine renal DHP-I than imipenem was.

**Systemic infection.** Because BO-3482 was susceptible to renal DHP-I, its efficacy against MRSA was initially investigated in the presence and absence of cilastatin. The ED₅₀ of BO-3482 alone and with cilastatin were 19.5 and 4.80 mg/kg, respectively, in the murine model of septicemia caused by the homogeneous strain MRSA BB6221 (Table 2). These results, together with the finding of vulnerability to DHP-I, showed that coadministration with cilastatin was necessary for BO-3482 to exert potent anti-MRSA activity in vivo (10). Therefore, cilastatin was used in the in vivo evaluation of BO-3482 against other S. aureus strains.

Table 2 also indicates the comparative protective efficacies of BO-3482–cilastatin, vancomycin, and imipenem-cilastatin against systemic MRSA and MSSA infections in normal mice. The ED₅₀ of BO-3482–cilastatin against homogeneous high-level MRSA BB6221 and BB6226 were 4.80 and 6.06 mg/kg,
respectively, while those of vancomycin were 5.56 and 2.15 mg/kg, respectively. BO-3482–cilastatin was as effective as vancomycin in protecting mice against the two homogeneous high-level MRSA strains, despite its lower in vitro activity (MICs, 6.25 versus 1.56 mg/ml). In these models, imipenem-cilastatin was ineffective (ED50, >200 mg/kg). BO-3482–cilastatin was as effective as vancomycin and was more effective than imipenem against heterogeneous low-level MRSA BB6156 infection; the ED50 of BO-3482–cilastatin was 0.46 mg/kg. Although the MIC of BO-3482 was eightfold higher than that of imipenem against MSSA Smith, there was no significant difference between BO-3482–cilastatin and imipenem-cilastatin. Similarly, BO-3482–cilastatin was also as active as vancomycin against the infection with MSSA Smith.

The protective effect of BO-3482 against MRSA BB6221 infection was investigated in mice with cyclophosphamide-induced immunosuppression (Table 3). There was no appreciable difference between the ED50 of BO-3482–cilastatin and vancomycin (3.15 versus 1.07 mg/kg).

**Thigh infection.** Figure 2 indicates the therapeutic effect of BO-3482 against thigh infection with MRSA BB6294 in immunosuppressed mice. With time after inoculation, the bacterial counts in the thigh muscle of the control animals showed a significant increase of 1.16 log10 CFU/thigh. The dose of BO-3482 used in this study was based on the possible maximum clinical dose. At all doses, BO-3482–cilastatin was more effective than vancomycin at 40 mg per kg.

BO-3482–cilastatin, vancomycin, and imipenem-cilastatin produced significant (*P < 0.001*) reductions in bacterial counts (2.34, 1.21, and 0.73 log10 CFU/thigh, respectively). Even at 10 mg per kg per dose, BO-3482–cilastatin caused a significant (*P < 0.001*) reduction in bacterial count of 1.36 log10 CFU/thigh, while vancomycin produced a nonsignificant reduction of 0.16 log10 CFU/thigh.

**TABLE 2. Protective efficacy of BO-3482 against systemic staphylococcal infections in normal mice**

<table>
<thead>
<tr>
<th>S. aureus strain, challenge dose (CFU/mouse [multiple of LD50])</th>
<th>Antibiotic</th>
<th>MIC (μg/ml)</th>
<th>ED50 (mg/kg) [95% confidence limit]a</th>
</tr>
</thead>
<tbody>
<tr>
<td>BB6221, 4.07 × 10^8 (4.4)</td>
<td>BO-3482</td>
<td>6.25</td>
<td>19.5 (9.51–44.3)</td>
</tr>
<tr>
<td></td>
<td>BO-3482–cilastatin^b</td>
<td>6.25</td>
<td>4.80 (1.66–10.7)</td>
</tr>
<tr>
<td></td>
<td>Vancomycin</td>
<td>1.56</td>
<td>5.56 (3.47–8.92)</td>
</tr>
<tr>
<td></td>
<td>Imipenem-cilastatin</td>
<td>100</td>
<td>&gt;200 (NC)</td>
</tr>
<tr>
<td>BB6226, 9.23 × 10^7 (13)</td>
<td>BO-3482–cilastatin</td>
<td>6.25</td>
<td>6.06 (2.58–14.7)</td>
</tr>
<tr>
<td></td>
<td>Vancomycin</td>
<td>1.56</td>
<td>2.15 (0.53–7.62)</td>
</tr>
<tr>
<td></td>
<td>Imipenem-cilastatin</td>
<td>100</td>
<td>&gt;200 (NC)</td>
</tr>
<tr>
<td>BB6156, 3.98 × 10^7 (3.5)</td>
<td>BO-3482–cilastatin</td>
<td>3.13</td>
<td>0.46 (0.05–2.17)</td>
</tr>
<tr>
<td></td>
<td>Vancomycin</td>
<td>1.56</td>
<td>1.79 (0.96–3.27)</td>
</tr>
<tr>
<td></td>
<td>Imipenem-cilastatin</td>
<td>12.5</td>
<td>15.9 (7.93–32.6)</td>
</tr>
<tr>
<td>Smith, 6.39 × 10^6 (29)</td>
<td>BO-3482–cilastatin</td>
<td>0.10</td>
<td>0.11 (0.063–0.194)</td>
</tr>
<tr>
<td></td>
<td>Vancomycin</td>
<td>0.20</td>
<td>0.31 (0.156–0.617)</td>
</tr>
<tr>
<td></td>
<td>Imipenem-cilastatin</td>
<td>0.012</td>
<td>0.052 (0.026–0.096)</td>
</tr>
</tbody>
</table>

^a Mice received a single subcutaneous dose of the antibiotic 1 h after bacterial challenge. The ED50 was expressed as the dose of each antibiotic.

^b Cilastatin was coadministered at 40 mg/kg.

^c NC, not calculated.

**TABLE 3. Protective efficacy of BO-3482 against systemic staphylococcal infections in immunosuppressed mice**

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>MIC (μg/ml)</th>
<th>ED50 (mg/kg/dose [95% confidence limit])</th>
</tr>
</thead>
<tbody>
<tr>
<td>BO-3482–cilastatin^b</td>
<td>6.25</td>
<td>3.15 (1.15–7.03)</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>1.56</td>
<td>1.07 (0.34–2.41)</td>
</tr>
<tr>
<td>Imipenem-cilastatin</td>
<td>100</td>
<td>34.5 (12.9–99.5)</td>
</tr>
</tbody>
</table>

^a Mice received subcutaneous doses of the antibiotic 1 and 3 h after bacterial challenge with 1.22 × 10^7 CFU/mouse (32 LD50).

^b Cilastatin was coadministered at 40 mg/kg.

FIG. 2. Comparative efficacies of BO-3482, vancomycin, and imipenem against MRSA in a murine thigh infection model. The right thighs of mice (n = 4) with cyclophosphamide-induced immunosuppression were injected subcutaneously with drugs 2 and 6 h after inoculation of homogeneous MRSA BB6294. The treatment regimens were 10, 20, or 40 mg of BO-3482 with 40 mg of cilastatin per kg per dose; 10, 20, or 40 mg of vancomycin (VCM) per kg per dose; or 40 mg of imipenem (IPM) per kg per dose. Four hours after the last treatment, the number of viable cells in the thigh muscles was determined and expressed as the geometric mean ± standard error. †, *P < 0.001 versus controls; *, *P < 0.001; **, *P < 0.01.
TABLE 4. Pharmacokinetic parameters of BO-3482 in mice*  

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>C_{max} (μg/ml)</th>
<th>t_{1/2} (min)</th>
<th>AUC_{Cmax} (μg.h/ml)</th>
<th>UR_{max} (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BO-3482</td>
<td>25.0</td>
<td>12.3</td>
<td>10.8</td>
<td>19.9</td>
</tr>
<tr>
<td>BO-3482-cilastatin</td>
<td>24.5</td>
<td>22.1</td>
<td>17.1</td>
<td>38.1</td>
</tr>
<tr>
<td>Imipenem-cilastatin</td>
<td>12.6</td>
<td>11.2</td>
<td>5.4</td>
<td>52.3</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>13.1</td>
<td>26.2</td>
<td>11.9</td>
<td>95.7</td>
</tr>
</tbody>
</table>

* Three mice per group were injected subcutaneously with a single dose of 10 mg/kg. AUC_{Cmax} = AUC from 0 to infinity; UR_{max} = urinary recovery rate from 0 to 6 h.

Cilastatin was coadministered at 40 mg/kg.

**Pharmacokinetic parameters.** The pharmacokinetic parameters of BO-3482 in mice are presented in Table 4. The C_{max} of BO-3482 given as a single subcutaneous dose of 10 mg/kg was higher than those of imipenem and vancomycin: C_{max} were 25.0, 12.6, and 13.1 μg/ml, respectively, even when BO-3482 was injected alone. Coadministration of BO-3482 with cilastatin extended the t_{1/2} in plasma, AUC, and urinary recovery of BO-3482 to levels 1.8-, 1.6-, and 1.9-fold higher than those for carbapenem given alone, respectively.

**DISCUSSION**

BO-3482, which has a dithiocarbamate structure at the C-2 position, is a novel β-methyl-carbapenem with potent anti-bacterial activity against MRSA. As reported previously (1, 7), BO-3482 has an improved affinity for PBP 2a (or PBP 2b) of MRSA, with a 50% inhibitory concentration of 3.8 μg/ml, which is approximately 30-fold higher than that of imipenem, which corroborates this anti-MRSA activity.

In the present study, the in vivo activity of BO-3482 against systemic infections caused by S. aureus strains with various levels of methicillin resistance was comparable to that of vancomycin, although the in vitro activity of BO-3482 was lower than that of vancomycin. Similarly, in a thigh infection model with a high-level MRSA strain, BO-3482 was more effective than vancomycin. This good in vivo efficacy of BO-3482 in combination with cilastatin may be ascribed to its higher C_{max} and t_{1/2} in plasma and higher AUC compared with those of imipenem in particular and also to the bactericidal nature of BO-3482, which is superior to that of vancomycin (1).

In conclusion, BO-3482 compared favorably with vancomycin in murine models of systemic and tissue infections caused by MRSA and appears to be a promising anti-MRSA agent.

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