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Therapeutic Efficacy of BO-3482, a Novel Dithiocarbamate Carbapenem, in Mice Infected with Methicillin-Resistant *Staphylococcus aureus*

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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 septicemia and thigh infection with methicillin-resistant *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 *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 antibiotic 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 *S. aureus* and be expressed by *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, (1*R*,5*S*,6*S*)-6-[(*R*)-1-hydroxyethyl]-2-{[*N*-(2-hydroxyethyl)-*N*-methyl amino] thiocarbonylthio}-1-methyl-1-carbapen-2-em-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 septicemia and thigh infection models.

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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 imipenem 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 *S. aureus* (MSSA) Smith were used for the murine septicemia model. MRSA BB6294 was used for the murine thigh infection model. MRSA BB6221, BB6226, and BB6294 are β-lactamase negative and homogeneously resistant, while strain BB6156 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 10^4 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

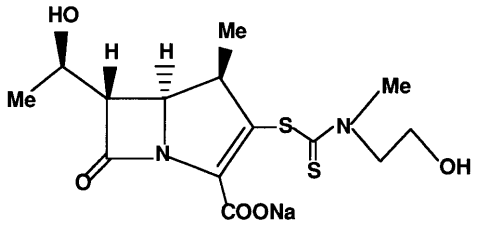

FIG. 1. Chemical structure of BO-3482. Me, methyl.

measuring the hydrolysis of glycyldehydrophenylalanine as a substrate. One unit of enzyme activity was defined as the amount of enzyme that hydrolyzed 1 μmol of a substrate per min 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 (ΔE) were as follows: BO-3482, 12.83 at 300 nm; imipenem, 9.04 at 299 nm. The Michaelis constant (K_m) and maximum rate (V_{max}) 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 mm^3) 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 (LD_{50}) 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 mg of the antibiotic per kg as the highest dose. Normal mice received a single dose of each antibiotic 1 h after infection, 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 (ED_{50}s) 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 BB6294 in tryptic soy broth (Difco) was washed and resuspended in fresh tryptic soy broth to ca. 10^8 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 injection 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 appropriate dilutions of the homogenate. The detection limit was 2 $\log_{10}$ CFU/ml for all antibiotics tested. The results obtained for each group were evaluated by calculating the arithmetic mean ± standard error. Statistical comparisons of viable bacterial counts for the different groups were performed by Fisher's protected least-significant-difference test (8). Data were considered significant when the P value was <0.05.

Pharmacokinetic study. Mice were injected subcutaneously with 10 mg of BO-3482 either alone or with 40 mg of cilastatin per kg of body weight. Imipenem was administered subcutaneously at a dose of 10 mg with 40 mg of cilastatin per kg (5). Vancomycin was injected subcutaneously at a dose of 10 mg per kg. Three mice per group were used for each time point. Blood samples were collected from three mice each at times of 5, 10, 30, and 60 min after administration. Blood was drawn from the heart and placed into heparinized tubes. Urine samples were collected from each of three mice kept in metabolic cages for 6 h after administration. The collected urine was pooled in an ice bath during the period of collection.

The concentrations of BO-3482 and imipenem in plasma and urine were determined by disk diffusion bioassay with antibiotic medium 1 (Difco) inoculated with *Bacillus subtilis* ATCC 12432 as the indicator organism (11), and those of vancomycin were determined by the same method but with 1% sodium citrate-supplemented nutrient agar (Difco) (15). Samples of plasma and urine were appropriately diluted with pooled mouse serum and 10 mM MOPS buffer (pH 7.0), respectively. A total of 26 μl of samples and standards were deposited 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 (C_{max}) was directly determined from the profiles of the concentration in plasma. The elimination rate constant (k_{el}) was calculated from linear regression analysis of the plasma concentration-time curve. The half-life ($t_{1/2}$) was calculated as $\ln 2/k_{el}$. 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 K_m and V_{max} 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 K_m values of murine and porcine DHP-Is for BO-3482 were 1/4 and 1/32 of those for imipenem, respectively. The relative V_{max} 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 V_{max}/K_m values of murine and porcine DHP-Is 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-Is 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 ED_{50}s 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 ED_{50}s of BO-3482–cilastatin against homogeneous high-level MRSA BB6221 and BB6226 were 4.80 and 6.06 mg/kg,

TABLE 1. Kinetic parameters of murine and porcine renal DHP-I in the hydrolysis of BO-3482 and imipenem

Antibiotic	Parameters of DHP-I derived from the following:					
	Mice			Swine		
	K_m (mM)	V_{max}[a]	V_{max}/K_m ratio[b]	K_m (mM)	V_{max}	V_{max}/K_m ratio
BO-3482	2.00	43.5	1.74	0.37	5.22	1.65
Imipenem	7.99	100	1.0	11.7	100	1.0

[a] Hydrolysis rate relative to that of imipenem when the hydrolysis rate of imipenem was taken as 100.
[b] Relative values when that for imipenem was taken as 1.0.

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

S. aureus strain, challenge dose (CFU/mouse [multiple of LD_{50}])	Antibiotic	MIC (μg/ml)	ED_{50} (mg/kg) [95% confidence limit][a]
BB6221, 4.07×10^8 (4.4)	BO-3482	6.25	19.5 (9.51–44.3)
	BO-3482–cilastatin[b]	6.25	4.80 (1.66–10.7)
	Vancomycin	1.56	5.56 (3.47–8.92)
	Imipenem-cilastatin	100	>200 (NC[c])
BB6226, 9.23×10^7 (13)	BO-3482–cilastatin	6.25	6.06 (2.58–14.7)
	Vancomycin	1.56	2.15 (0.53–7.62)
	Imipenem-cilastatin	100	>200 (NC)
BB6156, 3.98×10^7 (3.5)	BO-3482–cilastatin	3.13	0.46 (0.05–2.17)
	Vancomycin	1.56	1.79 (0.96–3.27)
	Imipenem-cilastatin	12.5	15.9 (7.93–32.6)
Smith, 6.39×10^6 (29)	BO-3482–cilastatin	0.10	0.11 (0.063–0.194)
	Vancomycin	0.20	0.31 (0.156–0.617)
	Imipenem-cilastatin	0.012	0.052 (0.026–0.096)

[a] Mice received a single subcutaneous dose of the antibiotic 1 h after bacterial challenge. The ED_{50} was expressed as the dose of each antibiotic.
[b] Cilastatin was coadministered at 40 mg/kg.
[c] NC, not calculated.

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 μg/ml). In these models, imipenem-cilastatin was ineffective (ED_{50}, >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 ED_{50} 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 ED_{50}s 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 $\log_{10}$ 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 $\log_{10}$ 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 $\log_{10}$ CFU/thigh, while vancomycin produced a nonsignificant reduction of 0.16 $\log_{10}$ CFU/thigh.

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

Antibiotic	MIC (μg/ml)	ED_{50} (mg/kg/dose [95% confidence limit])
BO-3482–cilastatin[b]	6.25	3.15 (1.15–7.03)
Vancomycin	1.56	1.07 (0.34–2.41)
Imipenem-cilastatin	100	34.5 (12.9–99.5)

[a] Mice received subcutaneous doses of the antibiotic 1 and 3 h after bacterial challenge with 1.22×10^7 CFU/mouse (32 LD_{50}s).
[b] Cilastatin was coadministered at 40 mg/kg.

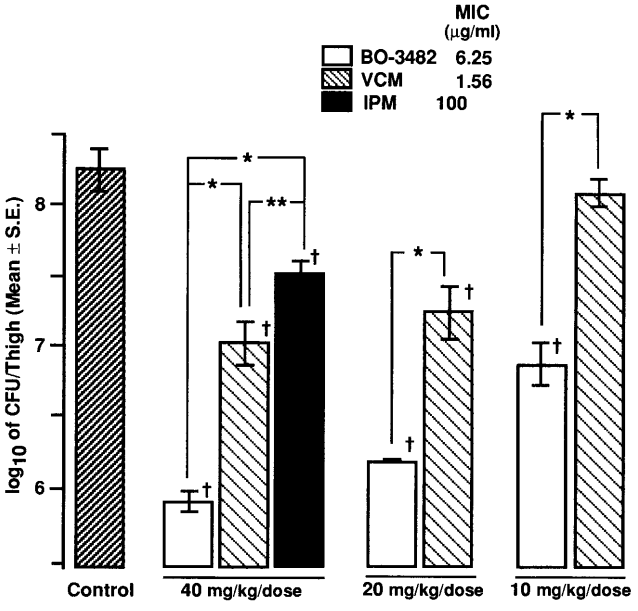

FIG. 2. Comparative efficacies of BO-3482, vancomycin, and imipenem against MRSA in a murine thigh infection model. The right thighs of mice ($n = 4$) with cyclophosphamide-induced immunosuppression were injected subcutaneously with drugs 2 and 6 h after inoculation of homogeneous MRSA BB6294. The treatment 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) with 40 mg of cilastatin 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[a]

Antibiotic	C_{max} (μg/ml)	$t_{1/2}$ (min)	$AUC_{0-\infty}$ (μg · h/ml)	UR_{0-6} (%)
BO-3482	25.0	12.3	10.8	19.9
BO-3482–cilastatin[b]	24.5	22.1	17.1	38.1
Imipenem-cilastatin	12.6	11.2	5.4	52.3
Vancomycin	13.1	26.2	11.9	95.7

[a] Three mice per group were injected subcutaneously with a single dose of 10 mg/kg. $AUC_{0-\infty}$, AUC from 0 h to infinity; UR_{0-6}, urinary recovery rate from 0 to 6 h.
[b] 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}s 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 1β-methyl-carbapenem with potent antibacterial activity against MRSA. As reported previously (1, 7), BO-3482 has an improved affinity for PBP 2′ (or PBP 2a) 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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