Patients with serious bacterial infections, such as nosocomial pneumonia, intra-abdominal infections, and complicated skin and soft tissue infections, are often treated empirically, because a delay in initiation of appropriate antimicrobial therapy has been shown to significantly increase morbidity and mortality (4, 11, 12, 21). The causative pathogens in these infections are a variety of gram-positive and gram-negative aerobes and anaerobes such as methicillin-resistant Staphylococcus aureus (MRSA) and Pseudomonas aeruginosa. MRSA is characterized by the expression of a special penicillin-binding protein (PBP) 2a. Its activity against laboratory mutants of P. aeruginosa with (i) overproduction of chromosomally coded AmpC β-lactamase; (ii) overproduction of the multidrug efflux pumps MexAB-OprM, MexCD-OprJ, and MexEF-OprN; (iii) deficiency in OprD; and (iv) various combinations of AmpC overproduction, MexAB-OprM overproduction, and OprD deficiency were tested. The increases in the MIC of tomopenem against each single mutant compared with that against its parent strain were within a fourfold range. Tomopenem exhibited antibacterial activity against all mutants, with an observed MIC range of 0.5 to 8 μg/ml. These results suggest that the antibacterial activity of tomopenem against the clinical isolates of MRSA and P. aeruginosa should be ascribed to its high affinity for PBP 2a and its activity against the mutants of P. aeruginosa, respectively.

Tomopenem (formerly CS-023) is a novel 1β-methylcarbapenem with broad-spectrum coverage of gram-positive and gram-negative pathogens. Its antibacterial activity against European clinical isolates of methicillin-resistant Staphylococcus aureus (MRSA) and Pseudomonas aeruginosa was compared with those of imipenem and meropenem. The MICs of tomopenem against MRSA and P. aeruginosa at which 90% of the isolates tested were inhibited were 8 and 4 μg/ml, respectively, and were equal to or more than fourfold lower than those of imipenem and meropenem. The antibacterial activity of tomopenem against MRSA was correlated with a higher affinity for the penicillin-binding protein (PBP) 2a. Its activity against laboratory mutants of P. aeruginosa with (i) overproduction of chromosomally coded AmpC β-lactamase; (ii) overproduction of the multidrug efflux pumps MexAB-OprM, MexCD-OprJ, and MexEF-OprN; (iii) deficiency in OprD; and (iv) various combinations of AmpC overproduction, MexAB-OprM overproduction, and OprD deficiency were tested. The increases in the MIC of tomopenem against each single mutant compared with that against its parent strain were within a fourfold range. Tomopenem exhibited antibacterial activity against all mutants, with an observed MIC range of 0.5 to 8 μg/ml. These results suggest that the antibacterial activity of tomopenem against the clinical isolates of MRSA and P. aeruginosa should be ascribed to its high affinity for PBP 2a and its activity against the mutants of P. aeruginosa, respectively.

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MATERIALS AND METHODS

Antibiotics. Tomopenem was synthesized at Daiichi Sankyo Research Laboratories, Tokyo, Japan (Fig. 1). IPM, MEM, and ceftazidime (CAZ) were obtained from the National Institute of Infectious Diseases, Tokyo, Japan. Levofloxacin was extracted at Daiichi Sankyo from a commercial formula. Amikacin (Banyu Pharmaceutical Co., Ltd., Tokyo, Japan) and oxacillin (Sigma-Aldrich Japan K.K.) were obtained commercially.

Bacterial strains. MRSA and P. aeruginosa used in the susceptibility tests were isolated from clinical specimens in European hospitals from 2001 to 2002 and from 2000 to 2002, respectively, and were stored frozen at −80°C. Sixty strains of MRSA were isolated in Germany, and 138 strains of P. aeruginosa were isolated in Germany (87 strains), France (19 strains), Italy (12 strains), Spain (7 strains), Ukraine (5 strains), Poland (4 strains), Russia (2 strains), Croatia (1 strain), and Lithuania (1 strain). S. aureus ATCC 29213 and P. aeruginosa ATCC 27853 were used as the quality control strains. The clinical isolates of MRSA 123-1 and 12386-1, which were isolated by culturing Japanese clinical isolates of MRSA 123...
and 12386 from which a penicillinase-encoding plasmid had been removed, were used for PBP 2a affinity tests. The *P. aeruginosa* mutants (N043, MR08, OCR1, N044, N045, N041, COR6, and N092) used in this study are described in Table 1. Highly CAZ-resistant mutant N043 was isolated by plating a CAZ-resistant mutant, which was isolated on Mueller-Hinton agar (MHA; Becton Dickinson and Company, Sparks, MD) containing 0.5 μg of CAZ per ml from PAO1, on MHA containing 50 μg of CAZ per ml. β-Lactamase produced in N043 was determined by UV spectrophotometry to have 80-fold more activity than that in a CAZ-resistant mutant. IPM-resistant mutants N041 and N044 were isolated by plating OCR1 and N043 on MHA containing 5 μg of IPM per ml, respectively. The outer membrane protein OprD was not detected in N041 and N044 by Western blot analysis with a rabbit anti-OprD antibody. Ofloxacin-MEM-resistant mutant N045 was isolated by plating N043 on MHA containing 0.8 μg of ofloxacin and 0.8 μg of MEM per ml. Ofloxacin-IPM-resistant mutant N092 was isolated by plating another PAO1 strain (a generous gift from T. Köhler) onto MHA containing 0.5 μg of ofloxacin and 1 μg of IPM per ml. A decrease in the amount of the outer membrane protein OprD and an increase in the amount of the outer membrane protein OprN in N092 were observed compared with the results seen with N091 by Western blot analysis with a rabbit anti-OprN antibody. Metallo-β-lactamase production was screened with modified Hodge and EDTA disk synergy tests (15).

**Susceptibility tests.** The MICs were determined by a standard microdilution broth method (23). Mueller-Hinton broth (Becton Dickinson and Company, Sparks, MD) containing 25 mg of CaCl₂ and 12.5 mg of MgCl₂ per liter (cation-adjusted Mueller-Hinton broth) was used. The inoculum size was 4 × 10⁶ CFU/ml. The MIC was defined as the lowest concentration of the compound that completely inhibited the visible growth of the organism in the microdilution wells. The determinations of the drug MIC against *P. aeruginosa* PAO1 and its mutants with various resistance mechanisms were performed in duplicate. Linear regression analysis (y = a + bx, where a represents the y intercept and b represents the slope) was used to correlate tompenem log₂ MICs (y) with IPM and MEM log₂ MICs (x). The correlation coefficient (r) was used to describe the scatter around lines of best fit. A statistical test was performed to assess the differences in the correlation coefficients (r). All P values were two-sided. The same analysis of Japanese *P. aeruginosa* isolates (9) was also performed.

**Affinity for PBP 2a.** The affinities of tompenem, IPM, and MEM for PBP 2a were determined by a competition assay using [³⁵S]benzylpenicillin (Amersham Japan Co., Ltd., Tokyo, Japan) as described previously (29, 31). Membrane fractions were collected by sequential centrifugations (5,000 × g for 10 min and 100,000 × g for 60 min) of enzymatically (100 μg of lysostaphin and 1 μg of DNase per ml) and sonically disrupted cells of MRSA 123-1 and MRSA 12386-1 in 50 mM sodium phosphate buffer containing 10 mM MgCl₂ (pH 7.0). The protein concentrations of the membrane fractions were adjusted to the final concentration of 8 mg/ml after protein quantitation with bovine serum albumin as the standard. The binding reactions were done for 30 min with test compounds at each concentration followed by 30 min with ³⁵S]benzylpenicillin at 37°C. The concentration required to prevent 50% of the binding of [³⁵S]benzylpenicillin (50% inhibition concentration [IC₅₀]) to PBP 2a were determined using a BAS 2000 imaging analyzer (Fuji Chemical Co. Ltd.).

### RESULTS

**Antibacterial activity of tompenem against MRSA and *P. aeruginosa***. Table 2 shows the antibacterial activity of tompenem against European clinical isolates of MRSA and *P.
The MICs at which 90% of the isolates tested were inhibited (MIC$_{90}$) of tomopenem, IPM, and MEM against 60 strains of MRSA were 8, >32, and 32 µg/ml, respectively. The MIC$_{90}$s of tomopenem, IPM, and MEM against 138 strains of *P. aeruginosa* were 4, 32, and 16 µg/ml, respectively. Scattergrams comparing the MICs of tomopenem with those of IPM and MEM for MRSA and *P. aeruginosa* are shown in Fig. 2.

Against MRSA, the MIC of tomopenem was twofold to fourfold higher than that of IPM when the MICs of tomopenem were less than or equal to 2 µg/ml against 16 strains out of 29 strains, while it was twofold to eightfold lower than that of IPM when the MICs of tomopenem were more than 2 µg/ml against 26 strains out of 31 strains. The MIC of tomopenem was equal to or more than twofold lower than that of MEM against the MRSA isolates tested, except for 1 strain. Tomopenem inhibited the growth of all *P. aeruginosa* strains, including IPM-resistant (MIC of IPM ≥ 16 µg/ml) and MEM-resistant (MIC of MEM ≥ 16 µg/ml) strains, at 8 µg/ml or lower. Tomopenem inhibited 98 percent of IPM-resistant *P. aeruginosa* strains (MIC of IPM ≥ 16 µg/ml), and 94% of MEM-resistant *P. aeruginosa* strains (MIC of MEM ≥ 16 µg/ml) showed MICs of tomopenem below 8 at 4 µg/ml or lower. Against European *P. aeruginosa* isolates, there was no significant difference ($P = 0.536$) between the activities of tomopenem and MEM (correlation coefficient, $r = 0.803$) and the activities of tomopenem and IPM (correlation coefficient, $r = 0.774$). Against Japanese *P. aeruginosa* isolates, the correlation between tomopenem and MEM (correlation coefficient, $r = 0.867$) was significantly higher ($P = 0.0290$) than that between tomopenem and IPM (correlation coefficient, $r = 0.765$). No metallo-β-lactamase producing strains were found (data not shown).

**Affinities of tomopenem for PBP 2a of MRSA.** Table 3 shows the affinities of tomopenem, IPM, and MEM for two strains of MRSA. The MIC of tomopenem against MRSA 123-1 and 12386-1 was 8 µg/ml, that of IPM and MEM against MRSA 123-1 was 32 µg/ml, and that of IPM and MEM against MRSA 12386-1 was 16 µg/ml. Tomopenem exhibited improved affinity which was more than 25-fold as high as that for IPM and more than 15-fold as high as that for MEM.

**Activity of tomopenem against *P. aeruginosa* with various resistance mechanisms.** Table 1 shows the MICs of tomopenem and comparators against *P. aeruginosa* PAO1 and its mutants with various resistance mechanisms. Against PAO1, a parent strain, the MIC of tomopenem was fourfold lower than that of IPM and twofold lower than that of MEM. Although...
the antibacterial activity of tomopenem against a mutant with deficiency in OprD (MR08), a double mutant with overproduction of β-lactamase and overproduction of MexAB-OprM (N045), a double mutant with deficiency in OprD and overproduction of MexAB-OprM (N041), and a double mutant with overproduction of β-lactamase and deficiency in OprD (N044) was reduced 4- to 32-fold, that of tomopenem against other mutants was almost the same as that against PAO1. Tomopenem exhibited antibacterial activity against all mutants, with an observed MIC range of 0.5 to 8 µg/ml.

**DISCUSSION**

Tomopenem showed antibacterial activity with MRSA and 
*P. aeruginosa* in European clinical isolates and laboratory mutants. The commercially available carbapenems such as IPM and MEM are insufficiently active against MRSA. Therefore, a number of carbapenems that target MRSA and other resistant gram-positive organisms have been investigated. However, so far carbapenems with both anti-MRSA activity and anti-*P. aeruginosa* activity have not been launched. Tomopenem exhibited improved activity against MRSA and *P. aeruginosa*.

There have been tomopenem phase II clinical trials conducted for complicated skin and skin structure infections in the United States and the European Union at doses of 750 mg three times a day (t.i.d.) and 1,500 mg t.i.d. J. L. Kutl et al. reported that the doses of 750 mg t.i.d. and 1,500 mg t.i.d. would achieve bactericidal exposures at breakpoints of 8 and 16 µg/ml, respectively, based on pharmacodynamic modeling (14). These proposed breakpoints would be differentiated clinically from existing carbapenems.

The MIC<sub>50</sub> of tomopenem and IPM against MRSA was 4 µg/ml, and the MIC<sub>50</sub>s of tomopenem and IPM were 8 and >32 µg/ml, respectively. According to the scattergrams, the MIC of tomopenem was equal to or more than twofold higher than that of IPM against most of the MRSA strains when the MICs of tomopenem were less than or equal to 2 µg/ml. However, it was equal to or more than twofold lower than that of IPM when the MICs of tomopenem were more than 2 µg/ml. The MIC of tomopenem was equal to or more than twofold lower than that of MEM against most of the strains. In this study, all the MRSA strains were isolated in Germany, and therefore this result may not be representative of Europe. We determined the IC<sub>50</sub> values for tomopenem, IPM, and MEM for PBP 2a with two strains of MRSA. The IC<sub>50</sub>s of tomopenem were more than 15-fold lower than those of IPM and MEM. Since the major mechanism of resistance to β-lactams in MRSA is the low affinity to PBP 2a, there would be a good correlation between the MIC of each carbapenem and the binding affinity for PBP 2a. This correlation is consistent with other new carbapenems (13, 35). The affinity of tomopenem for PBP 2a might be higher than that of IPM at the boundary of a MIC of tomopenem of more than 2 µg/ml. This hypothesis needs further evaluation, utilizing more strains with various MICs of tomopenem. The differences in MICs may be ascribed to the differences in the affinities for PBP 2a. However, the effects of other presumed factors influencing β-lactam resistance in MRSA, such as cell wall precursor formation and turnover, regulation, transport, and signal transduction (2), remain to be elucidated.

Recently, several cephalosporins and carbapenems that bind to PBP 2a with higher affinity than those of available β-lactams have been developed (7, 13, 32, 35). These compounds have in common a significantly longer side chain than commercially available β-lactams, which would be expected to increase their interactions with the active-site groove of PBP 2a (3, 16). The anti-MRSA activity of tomopenem may be related to structural aspects, such as a new side chain, a 2-guanidinoacyethylamo pyrrolidine moiety at position 2. Another group has also reported that the introduction of guanidine moieties to a pyrrolidine-3-thlthio group at the C-2 position in the carbapenem skeleton showed potent and well-balanced antibacterial activity, including anti-MRSA activity (24). This structural feature would permit the molecule to be positioned within the groove in such a way that the acylation reaction would proceed at a more rapid rate than that in commercially available carbapenems.

The emergence of carbapenem resistance in *P. aeruginosa* has become a global concern, since carbapenems remain important agents for the treatment of serious infections such as septicemia, pneumonia, and abdominal and urinary tract and skin and soft tissue infections due to multidrug-resistant *P. aeruginosa* in hospitalized patients and since only a few drugs are active against *P. aeruginosa*. Tomopenem showed more potent activity against *P. aeruginosa* from Europe, being four-fold more active than IPM and MEM. The correlation coefficient between MEM and tomopenem with European isolates was not significantly different from that between IPM and tomopenem. The increase in the drug MIC for the single mutant was similar to that of MEM in comparison with that of IPM (Table 1), and the affinity of tomopenem for PBPs 2 and 3 in *P. aeruginosa* ATCC 15692 was similar to that of MEM in comparison with IPM (N. Masuda, personal data). The reason for this lack of significant difference is unclear, but the proportion of MexA-MexB-OprM-overproducing OprD-deficient mutants in clinical isolates might be higher, since the increase in the drug MIC for this double mutant was similar to that of IPM in comparison with that of MEM. On the other hand, the correlation coefficient between MEM and tomopenem with Japanese isolates was significantly higher than that between IPM and tomopenem. The difference in analysis results between the European and Japanese isolates might be due to the difference in the proportions of the strains with various resistance mechanisms. Metallo-β-lactamase-producing *P. aeruginosa* was not detected in this study. The absence of metallo-β-lactamase-producing *P. aeruginosa* would reflect the region of isolates, since more than 60% of the strains were isolated from Germany, where metallo-β-lactamases are reported to be rare (8, 37).

The mechanisms associated with acquired resistance to carbapenems include the derepression of the chromosomal AmpC β-lactamases and loss of the substrate-specific channel, OprD, and the efflux pump, MexAB-OprM (17, 20). The MICs of tomopenem against the laboratory strains with these mechanisms were determined. Against PAO1, a parent strain, the MIC of tomopenem was equal to or more than twofold lower than those of IPM and MEM. The MR08 strain, an OprD-deficient mutant, showed increased resistance to all the carbapenems tested (Table 1), suggesting that, like other carbapenems, tomopenem penetrates outer membrane via this
channel. Against the OCR1 strain, a MexAB-OprM-overproducing strain, the increase in the MIC of tompopenem was twofold lower than that of MEM. This result suggests that tompopenem is a substrate for MexAB-OprM as well as MEM. Since these were single mutants for which the increases in the MICs of tompopenem and MEM were less than fourfold and eightfold, respectively, tompopenem could be considered to come under less influence from this kind of resistance mechanism than MEM. This expectation supported the observation that the MICs of tompopenem against laboratory mutants with combinations of resistance mechanisms whose strains were found in carbapenem-resistant isolates (N044, N045, and N041) were equal to or more than twofold lower than that of MEM. Against the strain N091, a MexEF-OprN-producing low-OprD-producing mutant, the increase in the MIC of tompopenem was twofold, although it is unclear whether tompopenem is a substrate for this pump or whether the association reflects the coregulation of MexEF-OprN with the low channel. Against the OCR1 strain, a MexAB-OprM-overproducing strain, the increase in the MIC of tompopenem was twofold lower than that of MEM. This result suggests that tompopenem is a substrate for MexAB-OprM as well as MEM. Since these were single mutants for which the increases in the MICs of tompopenem and MEM were less than fourfold and eightfold, respectively, tompopenem could be considered to come under less influence from this kind of resistance mechanism than MEM. This expectation supported the observation that the MICs of tompopenem against laboratory mutants with combinations of resistance mechanisms whose strains were found in carbapenem-resistant isolates (N044, N045, and N041) were equal to or more than twofold lower than that of MEM. Against the strain N091, a MexEF-OprN-producing low-OprD-producing mutant, the increase in the MIC of tompopenem was twofold, although it is unclear whether tompopenem is a substrate for this pump or whether the association reflects the coregulation of MexEF-OprN with the low

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