ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, July 1987, p. 1111–1116
0066-4804/87/071111-06$02.00/0

Vol. 31, No. 7

In Vitro and In Vivo Antibacterial Activities of T-2588, a New Oral Cephalosporin, Compared with Those of Other Oral β-Lactam Antibiotics

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Received 12 September 1986/Accepted 17 April 1987

T-2588, the pivaloyloxymethyl ester of T-2525, [6*R*, 7*R*]-7-[(*z*)-2-(2-aminothiazol-4-yl)-2-methoxyimino-acetoamido]-3-[(5-methyl-2H-tetrazol-2-yl)methyl]-3-cephem-4-carboxylic acid, is a new oral cephalosporin. T-2525 had a widely expanded antibacterial spectrum against gram-negative and gram-positive bacteria. T-2525 was more active in vitro than cefaclor, cephalexin, and amoxicillin against members of the family *Enterobacteriaceae* and *Branhamella catarrhalis*. Moreover, it exhibited superior in vitro activity against *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Neisseria gonorrhoeae*. T-2525 was highly stable to various β-lactamases, which were classified as Richmond and Sykes types Ia, Ib, Ic, III, IV, and Vc. It had high affinities for the lethal (essential) penicillin-binding proteins of *Escherichia coli*, *Clostridium perfringens*, and *Bacteroides fragilis*. T-2588 had excellent therapeutic effect on systemic infections in mice with various species of gram-negative bacteria, including β-lactamase-producing bacteria.

Oral cephalosporins such as cephalexin (1), cefaclor (1, 16), cefatrizine (17), cefadroxil (3), and cefroxadine (28) have been developed and used widely for clinical purposes. However, they have a relatively narrow spectrum against pathogens and are hydrolyzed by various types of β-lactamases. T-2588, the pivaloyloxymethyl ester of T-2525, is a new oral cephalosporin of the oxyimino type (Fig. 1). This ester type is equal to pivampicillin (4) and pivmecillinam (24). In this study, we compared the in vitro and in vivo antibacterial activity of T-2588 (T-2525) with those of cefaclor, cephalexin, and amoxicillin.

MATERIALS AND METHODS

Antibiotics. The antibiotics used in this study were T-2588, T-2525, cephalexin, ampicillin (Toyama Chemical Co., Ltd., Tokyo, Japan), cefaclor (Shionogi Pharmaceutical Co., Ltd., Osaka, Japan), amoxicillin (Beecham Pharmaceutical Co., Ltd., Tokyo, Japan), cephaloridine (Torii Pharmaceutical Co., Ltd., Tokyo, Japan), and benzylpenicillin (Meiji Seika Kaisya Ltd., Tokyo, Japan).

Bacterial strains. Standard strains were from culture collections in our laboratory. Clinical isolates of various species of bacteria were obtained from several hospitals in Japan.

Media. For preculture and MIC determination, sensitivity test broth (Nissui) and sensitivity test agar (Nissui) were used. However, media for particular species of bacteria were as follows. For *Streptococcus pyogenes*, brain-heart infusion broth (Difco Laboratories) and brain-heart infusion agar (Difco) were used. For *Streptococcus pneumoniae*, brain-heart infusion broth supplemented with 5% horse serum and brain-heart infusion agar supplemented with 5% horse blood were used. For *Haemophilus influenzae*, both brain-heart infusion broth and brain-heart infusion agar were supplemented with 1% hemin (Sigma Chemical Co.) and 0.2% β-nicotinamido-adenine dinucleotide (Sigma). For *Neisseria gonorrhoeae*, proteose no. 3 agar (Difco) supplemented with 1% hemoglobin (Difco) and 1% IsoVitaleX (Becton Dickinson Co.) was used. For obligate anaerobes, GAM broth (Nissui) and GAM agar (Nissui) were used.

Determination of MICs. The MICs were determined by the twofold agar dilution method. Precultures were incubated for 18 h at 37°C and suitably diluted in buffered saline containing 0.01% gelatine. One loopful (about 5 μl) of each diluted bacterial suspension was inoculated on drug-containing agar plates (1×10^4 to 4×10^4 CFU per spot) with a multiloop inoculator (Planter; Sakuma, Tokyo, Japan). Inoculated plates were incubated for 18 h at 37°C, except for obligate anaerobes, which were incubated for 48 h. *H. influenzae* and *N. gonorrhoeae* were incubated in a candle jar, and obligate anaerobes were incubated in an anaerobic globe box. The MIC was defined as the lowest drug concentration which prevented visible growth of bacteria.

Assay for PBPs. The affinity of T-2525 for penicillin-binding proteins (PBPs) was examined by a competition method described previously (5, 15, 18, 19, 22, 26). Various

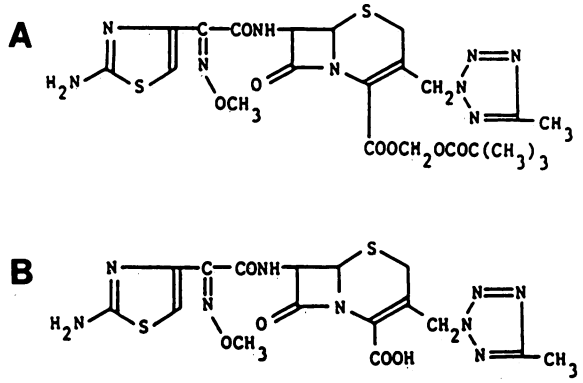

FIG. 1. Chemical structures of (A) T-2588 (pivaloyloxymethyl ester of T-2525) and (B) T-2525.

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TABLE 1. In vitro antibacterial activity of T-2525 against clinical isolates[a]

Organism (no. of isolates)	Antibiotic	MIC (μg/ml)[b] Range	50%	90%
Staphylococcus aureus (99)	T-2525	0.78–25	1.56	3.13
	Cefaclor	0.78–25	1.56	3.13
	Cephalexin	0.78–25	3.13	3.13
	Amoxicillin	0.1–25	0.78	1.56
Staphylococcus epidermidis (108)	T-2525	0.1–>200	3.13	50
	Cefaclor	0.03–100	3.13	25
	Cephalexin	0.03–>200	6.25	50
	Amoxicillin	≦0.01–50	0.78	12.5
Streptococcus pyogenes (91)	T-2525	≦0.01	≦0.01	≦0.01
	Cefaclor	0.05–1.56	0.1	0.39
	Cephalexin	0.1–0.78	0.39	0.78
	Amoxicillin	≦0.01–0.03	≦0.01	≦0.01
Streptococcus pneumoniae (24)	T-2525	≦0.01–0.03	≦0.01	0.03
	Cefaclor	0.39–0.78	0.39	0.78
	Cephalexin	1.56–3.13	3.13	3.13
	Amoxicillin	≦0.01–0.05	≦0.01	0.03
Clostridium perfringens (54)	T-2525	0.05–0.2	0.1	0.1
	Cefaclor	0.1–0.78	0.1	0.39
	Cephalexin	0.2–1.56	0.39	0.78
	Amoxicillin	≦0.01–0.03	≦0.01	0.03
Haemophilus influenzae (59)	T-2525	≦0.01–0.05	≦0.01	0.05
	Cefaclor	0.78–6.25	1.56	3.13
	Cephalexin	3.13–25	12.5	12.5
	Amoxicillin	0.39–200	12.5	100
Neisseria gonorrhoeae (24)	T-2525	≦0.01–0.39	0.05	0.1
	Cefaclor	0.2–50	3.13	12.5
	Cephalexin	0.05–50	3.13	25
	Amoxicillin	≦0.01–>200	0.78	>200
Branhamella catarrhalis (32)	T-2525	≦0.01–1.56	0.2	1.56
	Cefaclor	0.1–50	0.78	1.56
	Cephalexin	0.39–12.5	3.13	3.13
Escherichia coli (95)	T-2525	≦0.01–1.56	0.2	0.39
	Cefaclor	0.39–6.25	1.56	3.13
	Cephalexin	3.13–25	6.25	12.5
	Amoxicillin	0.78–>200	6.25	>200
Klebsiella pneumoniae (104)	T-2525	≦0.01–3.13	0.2	0.39
	Cefaclor	0.03–25	0.78	0.78
	Cephalexin	0.39–50	3.13	6.25
	Amoxicillin	0.78–>200	50	>200
Klebsiella oxytoca (73)	T-2525	0.03–3.13	0.1	0.78
	Cefaclor	0.2–>200	0.78	6.25
	Cephalexin	1.56–50	3.13	6.25
	Amoxicillin	3.13–>200	100	>200
Citrobacter freundii (76)	T-2525	0.1–>200	0.78	50
	Cefaclor	0.39–>200	6.25	>200
	Cephalexin	0.78–>200	25	>200
	Amoxicillin	0.78–>200	200	>200
Yersinia enterocolitica (52)	T-2525	0.05–6.25	0.39	0.39
	Cefaclor	1.56–>200	6.25	200
	Cephalexin	6.25–>200	12.5	200
	Amoxicillin	12.5–>200	100	100
Proteus mirabilis (99)	T-2525	0.03–0.2	0.05	0.1
	Cefaclor	0.39–12.5	1.56	1.56
	Cephalexin	6.25–50	12.5	25
	Amoxicillin	0.1–>200	0.78	1.56
Proteus vulgaris (84)	T-2525	≦0.01–6.25	0.1	0.2
	Cefaclor	0.39–>200	>200	>200
	Cephalexin	3.13–>200	>200	>200
	Amoxicillin	1.56–>200	>200	>200
Providencia rettgeri (55)	T-2525	≦0.01–12.5	0.05	0.39
	Cefaclor	0.2–>200	12.5	>200
	Cephalexin	0.78–>200	100	>200
	Amoxicillin	0.2–>200	50	>200
Providencia stuartii (99)	T-2525	≦0.01–12.5	0.2	0.78
	Cefaclor	0.1–>200	25	>200
	Cephalexin	0.2–>200	25	200
	Amoxicillin	0.2–>200	50	200
Morganella morganii (95)	T-2525	≦0.01–50	0.1	1.56
	Cefaclor	1.56–>200	>200	>200
	Cephalexin	12.5–>200	>200	>200
	Amoxicillin	25–>200	200	>200
Enterobacter cloacae (100)	T-2525	0.05–>200	3.13	100
	Cefaclor	0.78–>200	>200	>200
	Cephalexin	6.25–>200	>200	>200
	Amoxicillin	3.13–>200	>200	>200
Serratia marcescens (108)	T-2525	0.78–>200	3.13	100
	Cefaclor	12.5–>200	>200	>200
	Cephalexin	25–>200	>200	>200
	Amoxicillin	0.39–>200	>200	>200
Pseudomonas aeruginosa (100)	T-2525	12.5–>200	>200	>200
	Cefaclor	>200	>200	>200
	Cephalexin	>200	>200	>200
	Amoxicillin	25–>200	>200	>200
Bacteroides fragilis (33)	T-2525	1.56–>200	12.5	>200
	Cefaclor	25–>200	>200	>200
	Cephalexin	1.56–>200	25	>200
	Amoxicillin	3.13–>200	12.5	>200

[a] Agar dilution method.
[b] 50% and 90%, MIC for 50 and 90% of isolates, respectively.

concentrations of T-2525 (3 μl) and [^{14}C]penicillin G (0.15 μCi in 3 μl) (Amersham International; specific activity, 59 mCi/mmol) were added to the membrane proteins (20 mg/ml, 30 μl) prepared from *Staphylococcus aureus* 209P JC-1, *Escherichia coli* K-12 JE1011, *Clostridium perfringens* 13, and *Bacteroides fragilis* NCTC 9343 and incubated for 10 min at 30°C. The reaction was terminated by adding Sarkosyl and unlabeled penicillin G. ^{14}C-labeled PBP complexes were visualized after sodium dodecyl sulfate slab gel electrophoresis and fluorography. The extent of competitive inhibition of [^{14}C]penicillin G binding to PBPs by T-2525 was determined with a densitometer.

Stability to β-lactamases. Various types of β-lactamases used in this study were purified by methods described previously (6, 8, 10, 11, 13, 14, 25). These enzymes were purified by absorption and elution on a cellulose column, gel filtration on a Sephadex column, and so on from crude enzymes. Hydrolysis of drugs were assayed by a modifica-

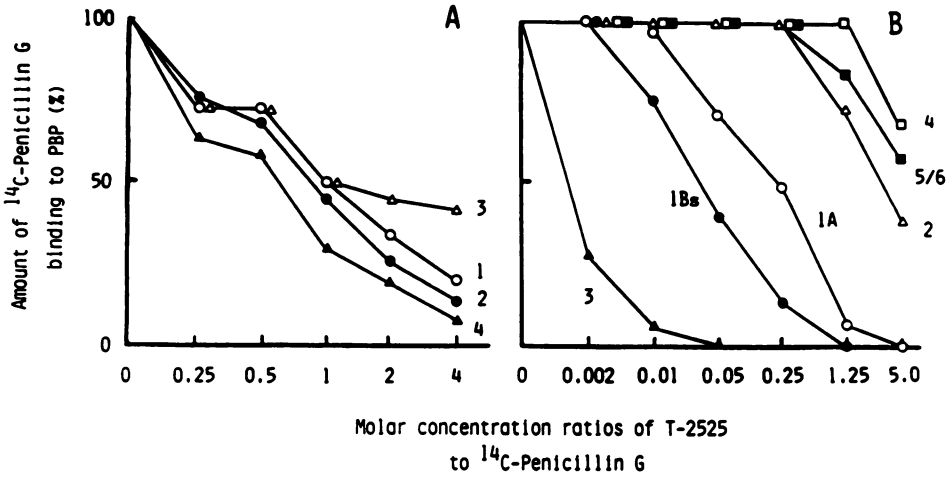

FIG. 2. Affinity of T-2525 to PBPs of *Staphylococcus aureus* 209P JC-1 (A) and *Escherichia coli* K-12 JE1011 (B). Relative amounts of [^{14}C]penicillin G were measured with a densitometer, with the amount of [^{14}C]penicillin binding without T-2525 set at 100%. MICs of T-2525 were 3.13 μg/ml for *S. aureus* 209P JC-1 and 0.2 μg/ml for *E. coli* K-12 JE1011.

tion of the microiodometric method (21), with penicillins as substrates, or by a spectrophotometric method (27), with cephalosporins as substrates, at a substrate concentration of 100 μM. The stability of the drugs to various β-lactamases was expressed as the relative rate of hydrolysis of substrate, with cephaloridine or benzylpenicillin representing 100% hydrolysis for penicillin and cephalosporin substrates, respectively.

Determination of in vivo activity. In vivo antibacterial activity was determined against systemic infections in mice. Ten 4-week-old male ICR strain mice weighing 19 to 21 g were used for each dose level. An overnight culture on heart infusion agar at 37°C was suspended in physiological saline or in 4% gastric mucin. A 0.5-ml volume of the bacterial suspension was inoculated intraperitoneally. The drugs were suspended in 0.5% carboxymethylcellulose and given as a single oral dose to the mice at 1 h after infection. The 50% effective dose (ED_{50}) was calculated by the probit method (2, 12) from the number of mice surviving for 7 days at each dose level after challenge. All untreated mice died within 24 h.

Serum and ascites drug concentrations in infected mice. Three or four 4-week-old male ICR strain mice weighing 19 to 21 g were used for each antibiotic. An overnight culture of *Klebsiella pneumoniae* Y-41 on heart infusion agar at 37°C was suspended in 4% gastric mucin. A 0.5-ml volume (10^2 CFU/0.5 ml) of the bacterial suspension was inoculated intraperitoneally. T-2588 and cephalexin were suspended in 0.5% carboxymethylcellulose and given as a single oral dose (50 mg/kg) to the mice at 1 h after infection. Serum and ascites samples were obtained from infected mice at 0.25, 0.5, 1, 2, 4, and 6 h after dosing. Serum and ascites drug concentrations were determined by a bioassay method. The test organism for T-2525 was *K. pneumoniae* ATCC 10031, and for cephalexin it was *Micrococcus luteus* ATCC 9341.

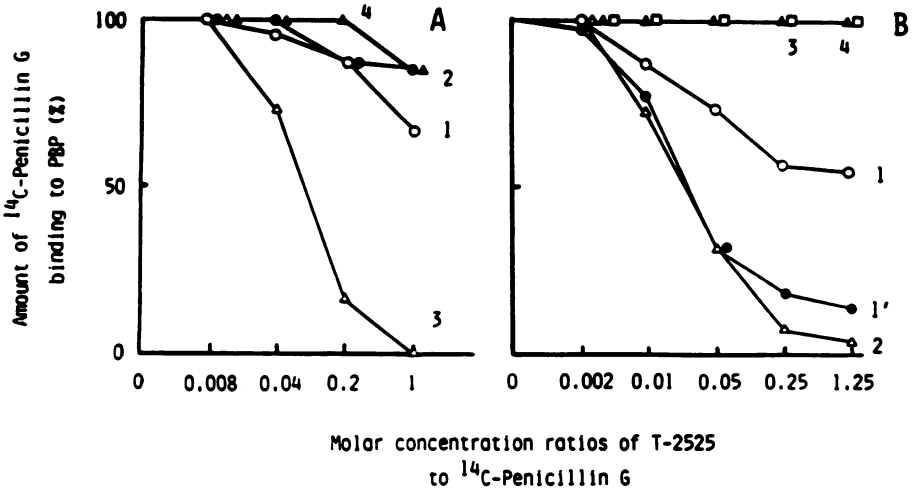

FIG. 3. Affinity of T-2525 to PBPs of *Clostridium perfringens* 13 (A) and *Bacteroides fragilis* NCTC 9343 (B). Relative amounts of [^{14}C]penicillin G were measured with a densitometer, with the amount of [^{14}C]penicillin binding without T-2525 set at 100%. MICs of T-2525 were 0.1 μg/ml for *C. perfringens* 13 and 6.25 μg/ml for *B. fragilis* NCTC 9343.

TABLE 2. Stability of T-2525 to β-lactamases

Enzyme source	β-Lactamase class[a]	Relative rate of hydrolysis[b] (% of control)					
		T-2525	CCL	CEX	APC	CER	PCG
Cephalosporinase							
Enterobacter cloacae H-27	Ia	0.2	106	35	0.6	100	4
Citrobacter freundii N-4	Ia	0.2	195	100	8.6	100	15
Serratia marcescens W-24	Ia	2.7	201	57	0.2	100	26
Providencia rettgeri GN4430	Ia	8.1	30	31	0.3	100	7.5
Escherichia coli GN5482	Ib	2.5	96	62	—[c]	100	—
Klebsiella oxytoca GN10650	Ic	1.9	33	5.0	286	100	—
Proteus vulgaris GN7919	Ic	1.1	34	20	29	100	7.0
Pseudomonas cepacia GN11164	Ic	2.1	292	45	165	100	73
Xanthomonas maltophilia GN12873 (L-2)	Ic	2.7	0.7	0.5	238	100	—
Bacteroides thetaiotaomicron GN11478	Ic	12	56	4.9	—	100	—
Penicillinase							
Escherichia coli ML1410/RTK-3	III (TEM)	0.1	1.9	0.2	—	17	100
Klebsiella pneumoniae Y-4	IV	0.2	4.1	0.4	126	15	100
Pseudomonas aeruginosa GN3379	Vc	0.1	0.1	0.1	95	58	100

[a] Based on the classification of Richmond and Sykes (23).
[b] Relative to hydrolysis rate for cephaloridine (cephalosporinase) or benzylpenicillin (penicillinase). Abbreviations: CCL, cefaclor; CEX, cephalexin; APC, ampicillin; CER, cephaloridine; PCG, benzylpenicillin.
[c] —, Not done.

RESULTS

Antibacterial activity against clinical isolates. The antibacterial activity of T-2525, cefaclor, cephalexin, and amoxicillin against clinical isolates is shown in Table 1. T-2525 was more active than cefaclor, cephalexin, and amoxicillin against *E. coli*, *Klebsiella* spp., and *Proteus mirabilis*. This drug was also active against other members of the *Enterobacteriaceae* which were not susceptible to the three reference drugs. Moreover, T-2525 had superior antibacterial activity against *S. pyogenes*, *S. pneumoniae*, *H. influenzae*, and *N. gonorrhoeae*. Especially, at the MIC for 90% of isolates, T-2525 was 64 times more active than cefaclor against *H. influenzae* and 128 times more active than cefaclor against *N. gonorrhoeae*.

Affinity for PBPs. The affinity of T-2525 for the PBPs of *S. aureus*, *E. coli*, *C. perfringens*, and *B. fragilis* was examined by measuring the competition of unlabeled T-2525 with [^{14}C]penicillin G for binding to PBPs. Figures 2 and 3 show the competition patterns quantitatively. T-2525 had remark-

TABLE 3. Protective effects of T-2588 against experimental infection in mice

Organism	Inoculum[a] (CFU/mouse)	Antibiotic	MIC[b] (μg/ml)	ED_{50}[c] (mg/kg)
Staphylococcous aureus Smith	1.00×10^7 (16)	T-2588	1.56	3.57 (1.26–9.27)
		Cefaclor	0.78	0.42 (0.24–0.69)
		Cephalexin	1.56	0.64 (0.38–1.02)
Escherichia coli ML4707	1.15×10^7 (43)	T-2588	0.1	1.07 (0.69–1.67)
		Cefaclor	0.78	2.66 (1.55–4.53)
		Cephalexin	3.13	30.1 (17.7–56.7)
Klebsiella pneumoniae Y-41	3.26×10^2 (326)	T-2588	0.39	6.05 (4.80–7.60)
		Cefaclor	1.56	8.75 (6.35–12.1)
		Cephalexin	12.5	56.0 (42.5–73.5)
Proteus mirabilis GN4754	1.16×10^7 (5)	T-2588	0.05	0.06 (0.02–0.15)
		Cefaclor	0.78	3.81 (1.99–6.02)
		Cephalexin	6.25	37.1 (23.6–63.1)
Serratia marcescens L-65	1.46×10^7 (61)	T-2588	0.78	0.27 (0.16–0.46)
		Cefaclor	>200	>500
		Cephalexin	>200	>500
Escherichia coli ML5005 (penicillinase-producing strain)	1.05×10^7 (2)	T-2588	0.2	0.79 (0.28–2.31)
		Cefaclor	3.13	24.0 (13.6–49.5)
		Cephalexin	6.25	117(81.4–158)
Escherichia coli 96 (cephalosporinase-producing strain)	1.10×10^7 (7)	T-2588	0.78	25.0 (16.7–38.0)
		Cefaclor	>200	>500
		Cephalexin	>200	>500

[a] Injected intraperitoneally with gastric mucin, except *E. coli* ML4707, which was injected without gastric mucin. Numbers in parentheses are the inoculum divided by the 50% lethal dose.
[b] Agar dilution method. MIC of T-2588 were measured by using T-2525.
[c] Numbers in parentheses are 95% confidence limits.

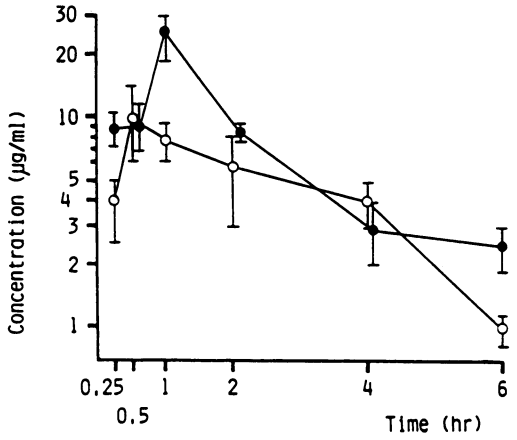

FIG. 4. Serum level of T-2525 and cephalexin after oral administration of T-2588 and cephalexin (50 mg/kg) to mice infected with *Klebsiella pneumoniae* Y-41. Symbols: ○, T-2525; ●, cephalexin. Data shown represent mean ± standard error.

ably high affinity for PBP 3 of *E. coli*. T-2525 had high affinities for PBP 1A and 1Bs of *E. coli*, PBP 3 of *C. perfringens*, and PBP 1, 1', and 2 of *B. fragilis*. T-2525 also had affinities for PBP 1, 2, 3, and 4 of *S. aureus*. The affinity of T-2525 for PBP 2, 4, and 5/6 of *E. coli*, PBP 3 and 4 of *B. fragilis*, and PBP 1, 2, and 4 of *C. perfringens* was low.

Stability to β-lactamase. The stability of T-2525 to β-lactamases is shown in Table 2. T-2525 was more stable than cefaclor and cephalexin to various β-lactamases, which were classified as Richmond and Sykes types Ia, Ib, Ic, III, IV, and Vc (23). T-2525 was slightly unstable to class Ic β-lactamase produced by *Bacteroides thetaiotaomicron*.

In vivo antibacterial activity. The protective effects of T-2588, cefaclor, and cephalexin on systemic infections in mice are shown in Table 3. T-2588 had superior activity against infections with *E. coli*, *K. pneumoniae*, *P. mirabilis*, and *Serratia marcescens*. Especially, with ED_{50} of T-2588 against *P. mirabilis* GN4754 infection was 0.06 mg/kg, and this agent was thus 64 times more active than cefaclor.

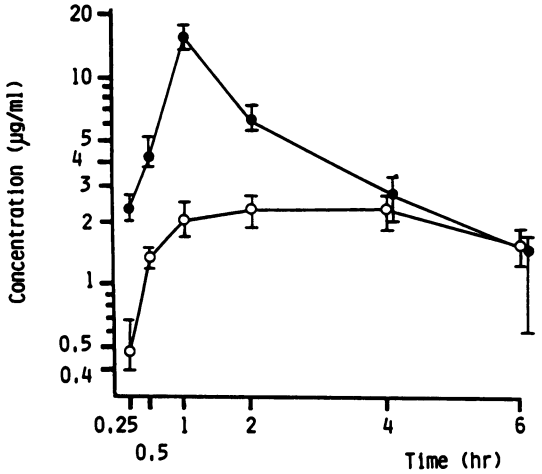

FIG. 5. Ascites level of T-2525 and cephalexin after oral administrations of T-2588 and cephalexin (50 mg/kg) to mice infected with *Klebsiella pneumoniae* Y-41. Symbols: ○, T-2525; ●, cephalexin. Data shown represent mean ± standard error.

Moreover, T-2588 was more active than the two reference drugs in infections with β-lactamase producing strains.

Serum and ascites drug concentrations in infected mice. Serum and ascites drug concentrations in infected mice are shown in Fig. 4 and 5. The concentration of T-2525 in the serum of infected mice was 9.8 μg/ml at the peak level. The concentration of T-2525 in the ascites of infected mice was 2.2 μg/ml at the peak level.

DISCUSSION

T-2588 is an orally administered pivaloyloxymethyl ester of T-2525. Metabolism of T-2588 was studied; it was absorbed through the upper intestine and hydrolyzed into T-2525 by esterases in the intestinal wall, and T-2525 was transferred into blood (J. Shimada, T. Saikawa, M. Tai, and H. Sadaki, Program Abstr. 25th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 584, 1985).

T-2525 had potent in vitro antibacterial activity against many clinical isolates. It had superior antibacterial activity against *S. pyogenes*, *S. pneumoniae*, *H. influenzae*, *N. gonorrhoeae*, and members of the *Enterobacteriaceae*. T-2525 was highly stable to various types of β-lactamase. T-2525 is an oxyimino-type cephalosporin such as cefuroxime (20) and cefotaxime (7). T-2525 had extremely high affinity to PBP 3 of *E. coli*. The dose of T-2525 that inhibited [^{14}C]penicillin-binding by 50% against PBP 3 of *E. coli* was more than 260 times lower than that of cephalexin (unpublished data). Moreover, T-2525 had high affinity for the killing-target proteins of *E. coli* (PBP 1A, 1Bs, and 3) (19, 26), *C. perfringens* (PBP 3 and 4) (15), and *B. fragilis* (PBP 1, 1', and 2) (9, 22) and also had affinity for same proteins of *S. aureus* (PBP 2 and 3) (5, 18). These results suggested vigorous bactericidal activity of T-2525 against various strains.

T-2588 had excellent therapeutic effects against systemic infections in mice with various species of gram-negative bacteria, including β-lactamase-producing bacteria. The pharmacokinetics of T-2588 was studied, and the serum half-life of T-2525 was longer than those of cefaclor and cephalexin in experimental animals (mouse, rat, and rabbit) (I. Saikawa, Y. Yasuda, Y. Watanabe, S. Minami, and H. Sadaki, 24th ICAAC, abstr. no. 225, 1984).

The data shown in this paper and the pharmacokinetics studies suggest that T-2588 would be useful in the clinical treatment of bacterial infections.

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