EXCELLENT ACTIVITY OF FK037, A NOVEL PARENTERAL BROAD-SPECTRUM CEPHALOSPORIN, AGAINST METHICILLIN-RESISTANT STAPHYLOCOCCI

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(Received for publication May 25, 1992)

FK037 exhibits potent *in vitro* and *in vivo* antibacterial activity against methicillin-resistant staphylococci. In *in vitro* studies, FK037 was the most active of the cephalosporins and imipenem tested against the highly methicillin-resistant staphylococci (MIC $>100\,\mu g/ml$). Only 2 of 57 strains of highly methicillin-resistant *Staphylococcus aureus* (H-MRSA) had a FK037 MIC value of $50\,\mu g/ml$. On the other hand, 55, 40 and 19 strains had MICs of 50 or $\geqq 100\,\mu g/ml$ to cefpirome, flomoxef and imipenem, respectively. Against 13 strains of highly methicillin-resistant coagulase-negative staphylococci (H-MRCNS), FK037 inhibited all the strains at $\leqq 50\,\mu g/ml$, but there were many strains highly resistant to the reference drugs with MICs of $\geqq 100\,\mu g/ml$. The influence of culture conditions such as low temperature, high inoculum and supplementation with 4% NaCl on the anti-MRSA activity of FK037 was less than those with cefpirome, flomoxef and imipenem. The *in vitro* frequency of spontaneous mutant cells highly resistant to FK037 in MRSA was lower than that to cefpirome and flomoxef. These findings were supported by lack of colonies inside the inhibition zone demarcated by FK037 in a disk sensitivity test, although many colonies proliferated inside the inhibition zone demarcated by flomoxef and imipenem. The increase in MIC of FK037 against a MRSA strain during subculture in the presence of the drug was smaller than that noted with the reference drugs. FK037 had higher affinity and faster binding for the PBP 2a of MRSA than that of the reference drugs. Moreover, the capacity to induce PBP 2a was lower for FK037 than that of cefpirome but higher than that of flomoxef. In an *in vitro* pharmacokinetic model simulating human plasma concentrations, FK037 showed potent bactericidal activity against H-MRSA in the plasma concentrations after intravenous infusion dosing with 1.0 g. FK037 was synergistically active against H-MRSA in combination with either imipenem of fosfomycin. The *in vitro* post-antibiotic effect (PAE) of FK037 against H-MRSA ranged from 1.2 to 1.7 hours at one to four times the MIC.

FK037 had potent therapeutic effects against lethal systemic infections and experimental local infections in mice such as pneumonia, endocarditis, subcutaneous abscess, intrauterine infection and granuloma pouch infection due to MRSA or methicillin-resistant *Staphylococcus epidermidis* (MRSE). FK037 was about 4, 8 and 1.5 times more effective than cefpirome, flomoxef and imipenem, respectively, against lethal systemic infections with H-MRSA. Against imipenem-resistant H-MRSA, FK037 was 3 to 11 times more effective than imipenem. Although the *in vitro* activity of FK037

was inferior to that of vancomycin, the ED_{50} ratio to those of vancomycin ranged from 1.2 to 3.4, which were less than those anticipated from the difference in MICs. Moreover, the therapeutic effect of FK037 was little influenced by challenge doses, while those of cefpirome and flomoxef were markedly reduced by high challenge doses. Against local infections due to MRSA or MRSE, FK037 was the most effective of the β-lactams tested. The therapeutic effect of FK037 was stronger than that anticipated based on its *in vitro* activity. Moreover, FK037 was as effective as vancomycin against MRSA in a subcutaneous abscess model. Against murine pneumonia with H-MRSA in an *in vivo* pharmacokinetic model simulating human plasma concentrations for intravenous infusion of 2.0 g, FK037 was significantly more effective than cefpirome and flomoxef in reducing the number of viable bacteria in the lungs. In granuloma pouch infection due to H-MRSA, the *in vivo* frequency of cells highly resistant to methicillin after 5 treatments with FK037 was lower than that with cefpirome and flomoxef. FK037 showed synergistic effect with imipenem or fosfomycin aginst lethal systemic infection with MRSA. FK037 produced *in vivo* PAE of 4.1 hours against a thigh infection with H-MRSA in neutropenic mice. From these results, FK037 merits further clinical trials for infection with MRSA.

FK037, a new parenteral cephalosporin, is regarded as the so-called fourth generation cephalosporin. It exhibits significant *in vitro* and *in vivo* activities against Gram-positive bacteria including highly methicillin-resistant staphylococci and Gram-negative bacteria including *Citrobacter* and *Enterobacter* resistant to the so-called third generation cephalosporins, and *Pseudomonas aeruginosa*[1,2]. Since methicillin-resistant *Staphylococcus aureus* (MRSA) have multiple drug resistance, the *in vitro* and *in vivo* anti-MRSA activities of FK037 were studied in an attempt to evaluate the clinical value of the drug in relation to a number of reference drugs.

Materials and Methods

Drugs

FK037 (lot 530198P, 520197P, 301186S, 510194P, 330189S) and cefpirome (lot 331199S, 331108S, 314176S) were synthesized in the New Drug Research Laboratories of Fujisawa Pharm. Co., Ltd., Osaka. The other drugs were commercially procured: Flomoxef (lot BC05, CDH01, FM8235) from Shionogi & Co., Ltd., Osaka; cefuzonam (lot 165-2, 117-1), cefotiam (lot OK136) and minocycline (lot 1178) from Takeda Chemical Ind., Ltd., Osaka; imipenem/cilastatin (imipenem, lot 6B003P, F200K, D087K, C014K, F177K, 6B028P) and methicillin (lot D023K, C022K) from Banyu Pharmaceutical Co., Ltd., Tokyo; ceftizoxime (lot 20017XG), cefazolin (lot 200194G) and vancomycin (lot 0800) from Fujisawa Pharm. Co., Ltd., Osaka; cefmetazole (lot K048L) from Sankyo Co., Ltd., Tokyo; and fosfomycin (lot F0SD1141) from Meiji Seika Kaisha, Ltd., Tokyo.

Bacteria

The strains studied were clinical isolates from various hospitals in Japan. *Staphyloccus aureus* 2562M2 was a methicillin-susceptible subclone isolated from *S. aureus* 2562 (low level MRSA, L-MRSA) during subcultivation in drug-free media, whereas *S. aureus* 2562HR was a MRSA subclone selected from the strain on agar media containing imipenem at 6.25 μg/ml. The criteria for drug resistance were methicillin MICs of 12.5 to 100 μg/ml for L-MRSA and low level methicillin-resistant coagulase-negative staphylococci (L-MRCNS) and >100 μg/ml for H-MRSA and highly MRCNS (H-MRCNS). Strains were grown on Mueller-Hinton agar containing 3.13 μg/ml methicillin at the first passage to avoid loss of methicillin-resistance, followed by growth on the same agar medium without drug. All strains used for experimental infections were previously screened for pathogenicity.

Animals

ICR-strain mice of either sex aged 4 or 5 weeks and SD-strain male rats aged 6 weeks were used for the experiments (Japan SLC Co.). Animals were housed in cages and food and water were given *at libitum*.

Determination of Minimum Inhibitory Concentration (MIC)

MIC values were determined by the agar dilution method on Mueller-Hinton agar (Difco). Strains were precultured in Trypticase soy broth (BBL). A hundred-fold diluted bacteria ($10^3 \sim 10^4$ cfu) were inoculated with a multipoint replicating apparatus onto agar plates containing serial 2-fold dilutions of each antibiotic prior to incubation at 37°C for 18 hours. The MIC was read as the lowest concentration of drug that inhibited visible growth of the organism. Growth of less than 5 colonies was considered negative. For evaluating the combination effects of FK037 with either imipenem or fosfomycin, the checkerboard agar dilution method was used. The combination effects were based on the fractional inhibitory concentration (FIC) index as synergistic (FIC index $\leqq 0.5$), indifferent ($0.5 <$ FIC index $\leqq 2.0$) or antagonistic (FIC index > 2.0)[3]. Factors such as high inoculum ($10^5 \sim 10^6$ cfu), low temperature (30°C) and supplementation with 4% NaCl were studied for their effects on MIC values.

Disk Sensitivity Test

Bacteria grown on Mueller-Hinton agar were suspended in saline and adjusted to 0.5 McFarland standard. A volume of 0.1 ml of the suspension was spread on the Mueller-Hinton agar plate with a glass bead. Disks (8 mm diameter, Toyo Roshi) containing 30 μg of drug were placed on the agar and the plates were incubated at 37°C for 48 hours. The diameter of the inhibition zone and the colonies inside the inhibition zone were observed.

In Vitro Frequency of Resistant Cells

Bacteria grown overnight in Trypticase soy broth were smeared onto a Mueller-Hinton agar plate containing drugs at concentrations of 1-, 2-, 4- or 8-times the MIC. The plates were incubated at 37°C for 48 hours. The numbers of colonies on the drug-containing plates were determined. The ratio of the number of colonies on the drug-containing plate to that on control plate was calculated as the frequency of resistant cells.

Increase in Resistance During Subcultivation

MIC determination was performed by the macrodilution broth method in a Mueller-Hinton broth. MRSA was subcultured daily for 3 days in Mueller-Hinton broth containing increasing concentrations of each drug. The inoculation in each transfer was made from the tube containing 1/4 the MIC of each drug.

Affinity to Penicillin-binding Proteins (PBPs)

PBP assays were performed by the modified method of SPRATT[4,5]. The *S. aureus* 2562 M2 and *S. aureus* 2562 cells were treated with 50 μl of lysostaphin (Sigma) at 30°C for 30 minutes prior to sonication with Model 200M (Kubota) and ultracentrifugation at $100{,}000 \times g$. The membrane fractions from *S. aureus* 2562 M2 were exposed to drugs at 30°C for 10 minutes, whereas those from *S. aureus* 2562 were exposed for 30, 60 or 120 minutes because of the slow binding rate of β-lactams to PBP 2a. ^{14}C-Labeled benzylpenicillin (^{14}C-PCG, specific activity at 58.5 mCi/mmol, Amersham) was used for the detection of unsaturated PBPs by pretreated drugs. The labeled PBPs except PBP 2a were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 10% acrylamide and 0.12% bisacrylamide, whereas PBP 2a was subjected to SDS-PAGE with 8% acrylamide and 0.06% bisacrylamide for dissociation from the PBP 2 band[6]. The gels were processed for fluorography and the concentrations of drugs which inhibited the ^{14}C-PCG binding to 50% of non-treated PBPs (I_{50}) were determined as the affinity of PBPs to drugs after scanning the X-ray film with a densitometer Model CS-930 (Shimadzu).

Induction of PBP 2a

Induction of PBP 2a in *S. aureus* 302 was tested by the method of UBUKATA[7]. The strain in the log-phase was exposed to drugs in Mueller-Hinton broth and incubated with shaking at 30°C for 6 hours. Membrane fractions wre prepared by ultracentrifugation after treatment with lysostaphin and sonication. After SDS-PAGE, PBP 2a was detected with a densitometer Model CS-930, and the relative values to PBP 2a induced by ceftizoxime at 1 μg/ml were calculated.

Bactericidal Activity in an *In Vitro* Pharmacokinetic Model

Overnight cultures in Mueller-Hinton broth were diluted in the same fresh media to a final concentration of 10^6 cfu/ml and incubated with shaking at 37°C for 1 hour. The bacteria were then exposed to drugs at estimated human plasma concentrations achieved after intravenous dosing of 0.125 to 2.0 g by the method of NISHIDA[8]. The human plasma concentrations of FK037 were calculated based on levels obtained from healthy volunteers receiving intravenous FK037 (0.125 g), whereas those of cefpirome, ceftazidime and flomoxef were referred to previously published data[9~12]. Viable cells were counted at various time intervals and adjusted by dilution factors.

Determination of *In Vitro* Post-antibiotic Effect (PAE)

Overnight cultures in Mueller-Hinton broth were diluted in the same fresh media to a final concentration of about 10^7 cfu/ml prior to incubation with shaking at 37°C for 1 hour. The bacteria exposed to drugs for 2 hours were diluted 500- or 1,000-fold with drug-free fresh media to remove the drugs before proceeding further. Viable cells were counted at various time intervals after removal of the drugs. The PAEs were calculated as the difference in time required for the number of antibiotic exposed and unexposed bacteria to increase by 1 $\log_{10}$ above the number at the time immediately after drug removal[13].

Lethal Systemic Infection

For strains used in the infection models (except *S. aureus* 9002), groups of 8 male 4 week-old mice were intraperitoneally inoculated with 0.5 ml of bacterial suspension in 5% gastric mucin to give about 1 minimum lethal dose (MLD). *S. aureus* 9002 was similarly inoculated in groups of 8 male mice immunosuppressed with intraperitoneal administration of cyclophosphamide at 200 mg/kg 4 days before infection. Infected mice were treated subcutaneously with serially diluted drugs one hour after infection. Mice infected with *S. aureus* 9002 were given drugs twice at post-infection 1 and 3 hours. The survival of the infected mice was observed for 3 to 5 days, and the 50% effective dose (ED_{50}) was determined from the final survival rates by the Probit method[14].

For studies on the combination effects of FK037 with either imipenem of fosfomycin, *S. aureus* 5027 and *S. aureus* 6004 were inoculated in normal mice as described above. The serially diluted drugs were given subcutaneously to the infected mice either alone or in combination one hour after infection. From ED_{50}s values of the drug alone and in combination, the fractional effective dose (FED) index was calculated by the following equation[15]:

$$\text{FED index} = \frac{ED_{50} \text{ of drug A in combination}}{ED_{50} \text{ of drug A alone}} + \frac{ED_{50} \text{ of drug B in combination}}{ED_{50} \text{ of drug B alone}}$$

The combination effect was judged from the FED index as synergistic (FED index $\leqq 0.5$), additive ($0.5 <$ FED index $\leqq 1.0$), indifferent ($1.0 <$ FED index $\leqq 2.0$) or antagonistic (FED index > 2.0).

Pneumonia

Groups of 5 male mice (age; 4 weeks) were immunosuppressed by intraperitoneal cyclophosphamide at 200 mg/kg 4 days before infection. Bacterial suspension (0.05 ml) of *S. aureus* 5027 (1.8×10^6 cfu) in saline were intranasally inoculated in the anesthetized mice. The infected mice were subcutaneously treated with the drugs at 20 mg/kg/administration 6 times post-infection (3, 6, 24, 32, 48 and 56 hours). The lungs were aseptically removed from the infected mice 3 days after infection, and the viable bacteria in the homogenized lungs were counted by a conventional plating method. The detection limit of viable bacteria was 10 cfu per lung in this model.

Bacterial Endocarditis

Endocarditis was induced by the method of SANTORO[16]. Groups of 5 or 6 male rats (age; 6 weeks) each were used for the experiments. After an incision was made in the surgically exposed right carotid artery of anesthetized rats, a polyethylene tube PE10 (Clay Adams) was inserted in the incision until it reached the left ventricle *via* the aortic valve. The tubing was then tightly secured in place, and the wound was closed. After an elapse of 24 hours, 0.5 ml of bacterial suspension of *S. aureus* 5126 (5.0×10^4 cfu) in

saline was intravenously inoculated in the animals. The infected rats were treated intravenously with 3 post-infection administrations (5, 24 and 32 hours) of 10 mg/kg of the drugs. The hearts were aseptically removed from the infected rats 2 days after infection, and the viable bacteria in the homogenized heart were counted by a conventional plating method. The detection limit of viable bacteria was 10 cfu per heart in this model.

Subcutaneous Abscess

Groups of 7 male mice each were immunosuppressed with intraperitoneal cyclophosphamide at 200 mg/kg. On the fourth day, the animals were inoculated subcutaneously with either 0.1 ml of *S. aureus* 9027 (1.2×10^3 cfu) or *S. epidermidis* 9024 (4.4×10^6 cfu) on their dorsal regions. The infected mice were subcutaneously treated with 5 administrations of 10 mg/kg/administration (*S. aureus* 9027) post-infection (5, 24, 32, 48 and 56 hours) and 80 and 20 mg/kg/administration (*S. epidermidis* 9024) of the drugs at sites remote from the infection sites. Pieces of shaved skin of the animals at the infection sites were aseptically removed 3 days after infection, and the viable bacteria were counted after homogenization by a conventional plating method. The detection limit of viable bacteria was 10 cfu per piece of skin in this model.

Intrauterine Infection

Intrauterine infection was induced by the method of OBANA[17]. Groups of 5 female mice (age; 5 weeks) each were anesthetized and the uterus was surgically exposed. To prevent bacterial flux, the cervix was ligated with a surgical suture, taking care not to ligate the arteries. A volume of 0.025 ml of the bacterial suspension of *S. aureus* 5027 (5.5×10^5 cfu) was inoculated in the left uterine horns. The infected mice were subcutaneously treated with 5 administrations of 80 and 20 mg/kg/administration of the drugs at 5, 25, 32, 48 and 56 hours post-infection. The uterines were aseptically removed from the infected mice 3 days after infection, and the viable bacteria were counted by a conventional plating method after homogenization. The detection limit of viable bacteria was 10 cfu per uterine in this model.

Granuloma Pouch Infection

Granuloma pouch infection was induced by the method of NISHIDA[18]. Groups of 4 or 5 rats (age; 6 weeks) each were subcutaneously injected with 20 ml of air in their backs and 1 ml of olive oil containing 1% croton oil was injected into the pouch of each animal to induce aseptic inflammation. Six days later, a bacterial suspension of *S. aureus* 5027 (4.4×10^7 cfu) in 5% gastric mucin (2 ml) was inoculated into the granuloma pouch. The infected rats were treated intramuscularly with the drugs at post-infection, 5, 24, 32, 48 and 56 hours. Exudate fluid was removed from the granuloma pouch immediately, 24, 48 and 72 hours after infection, and the viable bacteria were counted by a conventional plating method. The detection limit of viable bacteria was 10 cfu per ml of the exudate fluid in this model.

In Vivo Frequency of Resistant Cells

Exudate fluids removed from the granuloma pouch 72 hours after infection of non-treated rats and rats treated with 20 mg/kg of the drugs were spread onto a Mueller-Hinton agar plate containing 200 μg/ml of methicillin and with no methicillin added. The plates were incubated 37°C for 24 hours and the numbers of colonies were determined. The ratio of the number of colonies on the drug-containing plate to that on control plate was calculated as the frequency of highly methicillin-resistant cells.

In Vivo Pharmacokinetic Model

1) Pneumonia: Groups of 5 male mice aged 4 weeks were immunosuppressed with 200 mg/kg cyclophosphamide (ip) 4 days before infection. Volumes of 0.05 ml of the bacterial suspension of *S. aureus* 5027 (2.6×10^6 cfu) in saline were intranasally inoculated in the anesthetized mice.

2) Doses and dosage regimens: The doses and dosing schedules of the drugs were determined to approximate the time-related concentration in human plasma produced by usual clinical treatment of drip infusion with 1.0 g over a 1-hour period. Since drip infusion was impossible in mice and their plasma clearance was much faster than that of human, frequent subcutaneous injections at a 1-hour intervals were necessary. Human plasma concentrations of FK037 after 2.0 g drip-infusion were calculated from those after drip infusion of 1.0 g[9]. Those of cefpirome and flomoxef were referred to data reported

previously[10,12]. Sixteen hours after the inoculation of bacteria, the mice were treated with the drugs according to the above dosage regimens.

3) Measurement of bacterial counts: Immediately before, 3, 6 and 24 hours after initial dosing, groups of 5 mice were sacrificed, and the lungs were aseptically removed. Each lung was homogenized and the number of viable bacteria was quantitated by a conventional plating method. The detection limit was 10 cfu per lung.

In Vivo Post-antibiotic Effect (*In Vivo* PAE)

In vivo PAE was studied by the method of GUDMUNDSSON[19]. Groups of 5 male mice each were immunosuppressed with intraperitoneal cyclophosphamide at 200 mg/kg and inoculated on the fourth day with 0.1 ml of *S. aureus* 5027 (1.7×10^6 cfu) in the thigh of each mouse. Two hours later (time 0), a dose of 80 mg/kg of FK037 was subcutaneously injected in the treated groups. The infected mice were killed at selected intervals from -2 to 12 hours. Thighs were aseptically removed, and the viable bacteria were counted by a conventional plating method after homogenization. The drug concentrations in the thighs infected with *S. aureus* 5027 after subcutaneous dosing of FK037 at 80 mg/kg were determined by the disc-agar diffusion method using *Morganella morganii* IFO 3848.

The PAE was calculated by the following equation: $PAE = T - C - M$, where M is the time thigh levels exceeded the MIC; T is the time required for a mean cfu/thigh of treated mice to increase to 1 $\log_{10}$ above the count at time M; and C is the time needed for the cfu/thigh of control animals to increase to 1 $\log_{10}$.

Statistics

The parallel line assay was used to determine the significant differences between FK037 and reference drugs in ED_{50}s for lethal systemic infections. Tukey- or Dunnett-type multiple comparison was used to determine the significant differences between the FK037-treated and reference drug-treated groups, and between the drug-treated and non-treated groups in $\log_{10}$ values of viable counts in local infections.

Results

Antibacterial Activity of FK037 against Methicillin-resistant Staphylococci

The activity of FK037 against clinical isolates of methicillin-resistant staphylococci was compared with that of cefpirome, flomoxef, cefuzonam, imipenem, fosfomycin or cefoperazone (Table 1). The MIC_{90}s of FK037 against 58 strains of L-MRSA and 57 strains of H-MRSA were 12.5 and 25 μg/ml, respectively. Against L-MRSA, FK037 was similar or inferior to flomoxef and imipenem, and was more potent than cefpirome, cefuzonam and fosfomycin. Against H-MRSA, FK037 was the most active of the drugs tested. The MIC_{90}s of FK037 against 21 strains of L-MRCNS and 13 strains of H-MRCNS were 6.25 and 50 μg/ml, respectively. Against L-MRCNS, FK037 was similar to cefpirome, flomoxef and cefuzonam, and was more potent than cefoperazone. Against H-MRCNS, FK037 was also the most active of all the drugs tested. As can be noted from the susceptibility distributions of the H-MRSA (57 strains) and H-MRCNS (13 strains), there were no highly resistant strains, MICs $\geqq 100$ μg/ml, to FK037 (Figs. 1a and 1b). On the other hand, there were many highly resistant strains to cefpirome, flomoxef and imipenem with MIC $\geqq 100$ μg/ml. In particular, only 2 of 57 H-MRSA strains had a FK037 MIC value of 50 μg/ml in contrast to the 55, 40 and 19 strains for cefpirome, flomoxef and imipenem, respectively.

Influence of Culture Conditions on Anti-MRSA Activity of FK037

The activity of FK037 and the reference drugs against 17 strains of L-MRSA under conditions of low temperature (30°C), Mueller-Hinton agar supplemented with 4% NaCl and high inoculum were compared with that under ordinary conditions (37°C, 10^{-2} inoculum, Mueller-Hinton agar) (Fig. 2). The mean MICs of FK037 under the 3 conditions were less than twice of those in ordinary condition, which

Table 1. Antibacterial activity of FK037 and reference drugs against methicillin-resistant staphylococci.

Organism (No. of strains)	Antibiotic	MIC (μg/ml)		
		Range	50%	90%
S. aureus [L-MRSA[a]] (58)	FK037	1.56~25	6.25	12.5
	Cefpirome	0.78~100	12.5	50
	Flomoxef	0.78~25	3.13	12.5
	Cefuzonam	0.78~100	12.5	100
	Imipenem	0.05~12.5	0.2	6.25
	Fosfomycin	3.13~>100	25	>100
S. aureus [H-MRSA[b]] (57)	FK037	6.25~50	25	25
	Cefpirome	25~>100	100	100
	Flomoxef	12.5~>100	50	100
	Cefuzonam	25~>100	>100	>100
	Imipenem	6.25~100	25	50
	Fosfomycin	6.25~>100	>100	>100
Coagulase-negative staphylococci [L-MRCNS[c]] (21)	FK037	1.56~12.5	3.13	6.25
	Cefpirome	0.78~25	3.13	12.5
	Flomoxef	0.78~12.5	3.13	6.25
	Cefuzonam	0.78~25	3.13	6.25
	Cefoperazone	1.56~>100	6.25	25
Coagulase-negative staphylococci [H-MRCNS[d]] (13)	FK037	3.13~50	25	50
	Cefpirome	12.5~>100	50	100
	Flomoxef	12.5~100	50	100
	Cefuzonam	6.25~>100	>100	>100
	Imipenem	3.13~>100	50	100

Agar dilution method (stamp method): Mueller-Hinton agar, 10^4 cfu/spot, 37°C, 18 hours.

[a] L-MRSA: Low level methicillin-resistant *Staphylococcus aureus* (methicillin MIC: 12.5~100 μg/ml).

[b] H-MRSA: Highly methicillin-resistant *Staphylococcus aureus* (methicillin MIC: >100 μg/ml).

[c] L-MRCNS: Low level methicillin-resistant coagulase-negative staphylococci (methicillin MIC: 12.5~100 μg/ml).

[d] H-MRCNS: Highly methicillin-resistant coagulase-negative staphylococci (methicillin MIC: >100 μg/ml).

was less than those obtained with cefpirome, flomoxef and imipenem under the same cultural conditions.

In Vitro Synergistic Effect of FK037 with either Imipenem or Fosfomycin against H-MRSA

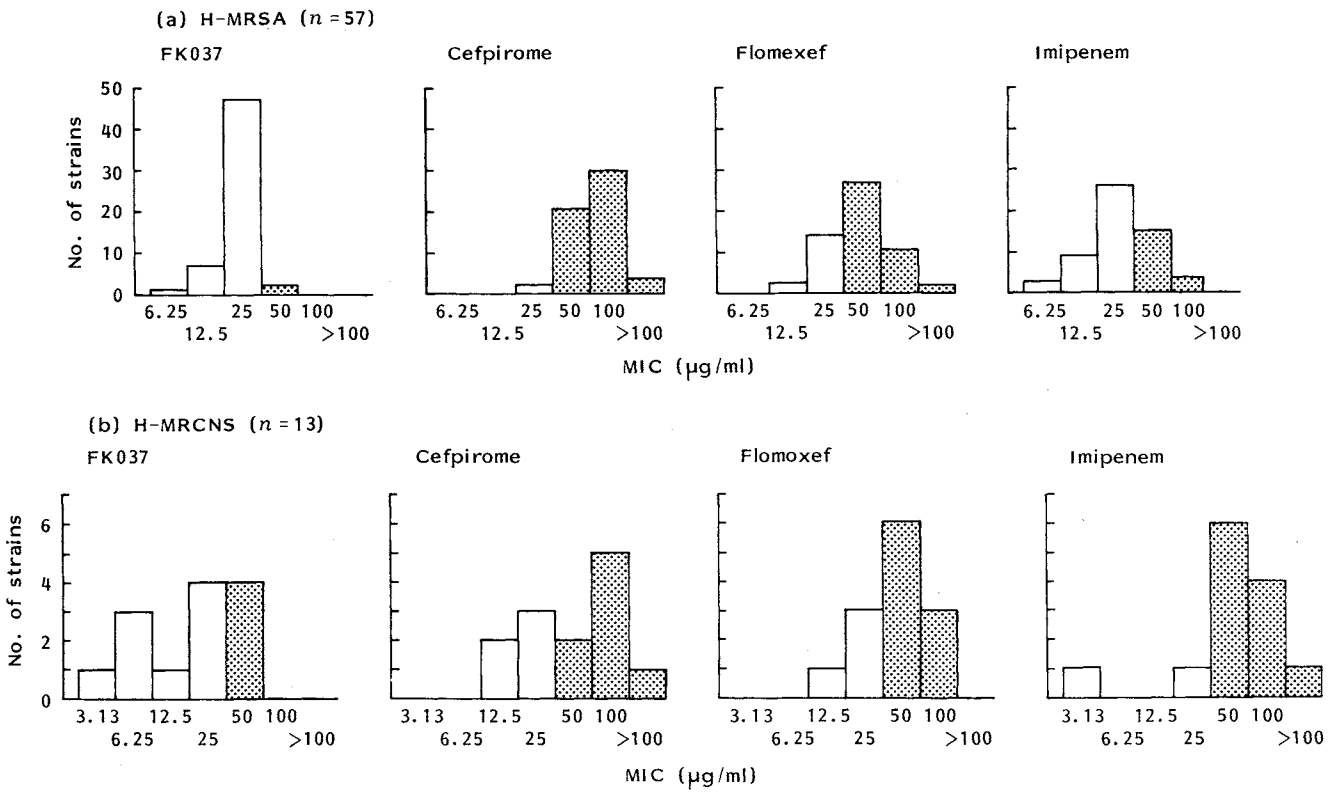
The mean FIC indexes, and the mean MICs of FK037, imipenem and fosfomycin alone and in combination are given in Table 2. The mean indices with the FK037/imipenem and FK037/fosfomycin combinations were 0.28 and 0.45, respectively. 90% or 75% of 20 H-MRSA strains were synergistically inhibited by the FK037 combination with either imipenem or fosfomycin, respectively.

In Vitro Frequency of Highly Resistant Cells to FK037

MRSA is usually heterogeneous in methicillin-resistance and a strain contains a few highly resistant cells[20]. The occurrence of colonial growth by such highly resistant cells inside the inhibition zone produced by FK037 was compared with those produced by flomoxef and imipenem in disk sensitivity test. Fig. 3 shows a representative photograph. The inhibition zone obtained with FK037 was clear cut with no colonies, however, there were many colonies within the inhibition zones of flomoxef and imipenem. The cells which grew inside the inhibition zones of flomoxef and imipenem were highly resistant to both drugs although their parent strain, *S. aureus* 8016 (L-MRSA), was susceptible to both drugs (Table 3). FK037 had comparable activity against both the resistant cells and the parent strain. Similar results were also found with 14 other MRSA strains.

The frequency of highly resistant cells capable of growth in the presence of FK037 at concentrations

Fig. 1. Susceptibility distribution of highly methicillin-resistant staphylococci to FK037 and reference drugs.



H-MRSA, H-MRCNS; See footnote to Table 1.

Table 2. *In vitro* combination effects of FK037 with either imipenem or fosfomycin against 20 strains of highly methicillin-resistant *Staphylococcus aureus*[a].

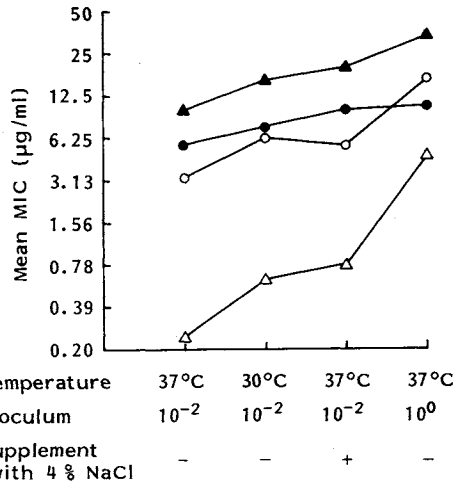
Antibiotic	Mean MIC (μg/ml)	Mean FIC index	% Synergism
FK037 alone	12.5		
Imipenem alone	8.84		
FK037/Imipenem combination	2.13/0.34	0.28	90.0
FK037 alone	14.9		
Fosfomycin alone	>800		
FK037/Fosfomycin combination	2.72/132	0.45	75.0

Checkerboard agar dilution method (stamp method): Mueller-Hinton agar, 10^4 cfu/spot, 37°C, 18 hours.

[a] See footnote to Table 1.

Fig. 2. Effect of incubation temperature, inoculum size and NaCl supplementation on antibacterial activity of FK037 and reference drugs against low level methicillin-resistant *Staphylococcus aureus* ($n=17$).

● FK037, ▲ cefpirome, ○ flomoxef, △ imipenem.



L-MRSA; See footnote to Table 1.

Fig. 3. Inhibition zones of FK037 and reference drugs against a highly methicillin-resistant *Staphylococcus aureus* 9079.

A: FK037 (30 μg/disc), B: flomoxef (30 μg/disc), C: imipenem (30 μg/disc).

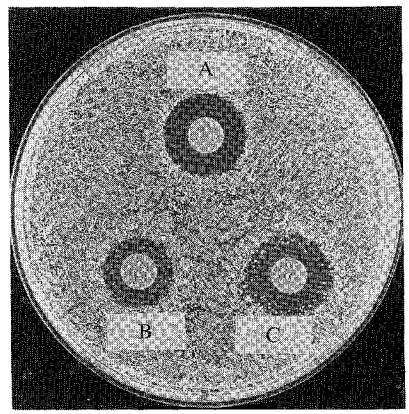


Table 3. Antibacterial activity of FK037 and reference drugs against cells which grew inside the inhibition zones of flomoxef and imipenem against low level methicillin-resistant *Staphylococcus aureus*[a].

Organism	Antibiotic	Mean MIC (μg/ml)		
		Parent	Flomoxef^R	Imipenem^R
S. aureus 8016	FK037	12.5	19.8	12.5
	Flomoxef	6.25	100	63.0
	Imipenem	0.78	63.0	39.7

Flomoxef^R, Imipenem^R: Flomoxef-, Imipenem-resistant cells ($n=2\sim3$) which grew inside the inhibition zones of flomoxef and imipenem.

[a] See footnote to Table 1.

Fig. 4. Incidence of spontaneous high resistance to FK037 and reference drugs in methicillin-resistant *Staphylococcus aureus* 5031.

● FK037 (MIC: 6.25 μg/ml), ▲ cefpirome (3.13 μg/ml), ○ flomoxef (1.56 μg/ml).

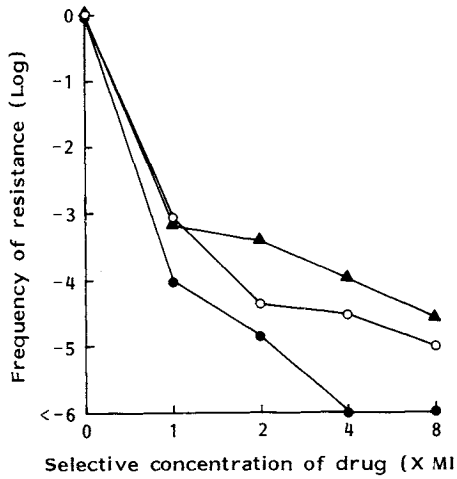
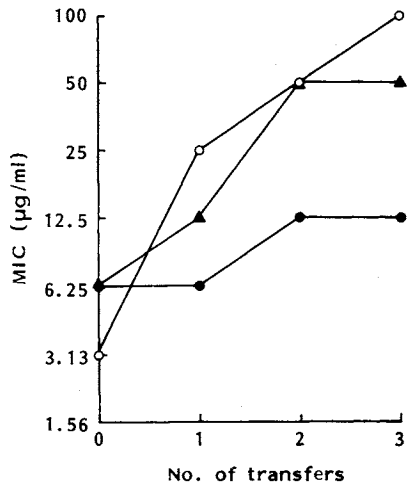


Fig. 5. Increase in resistance of methicillin-resistant *Staphylococcus aureus* 5031 by *in vitro* transfer in the presence of FK037 and reference drugs.

● FK037, ▲ cefpirome, ○ flomoxef.



of 1 MIC or higher was compared with that of cefpirome and flomoxef in *S. aureus* 5031 (L-MRSA) (Fig. 4). The incidence of the strain resistant to FK037 at 1 MIC was about 10^{-4} and was less than 10^{-6} at 4 MIC or more. On the other hand, the frequency of resistance to cefpirome or flomoxef was higher at all concentrations tested and were about 10^{-5} even at 8 MIC.

Increase of FK037-resistance by *In Vitro* Transfer

To confirm the influence of low incidence of highly resistant cells to FK037 on antibacterial activity, changes in the MIC against *S. aureus* 5031 during *in vitro* transfer in the presence of FK037 were compared with those in the presence of either cefpirome or flomoxef (Fig. 5). The MIC of FK037 against the strain was only 2-fold the initial MIC after 3 times-transfer. However, 8 and 32-fold increases in the initial MICs were obtained with cefpirome and flomoxef, respectively, values which were markedly higher than that of FK037.

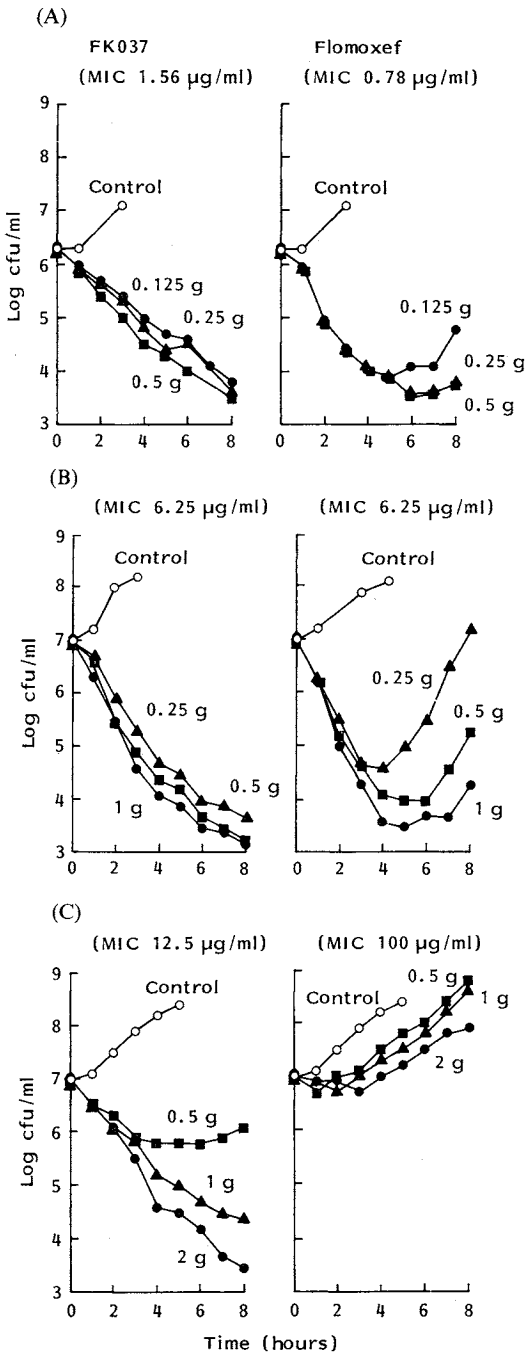
Bactericidal Activity of FK037 in an *In Vitro* Pharmacokinetic Model

Bactericidal activity of FK037 against *S. aureus* 2562 HR (a H-MRSA subclone selected from *S. aureus* 2562 on agar media containing imipenem at 6.25 μg/ml), *S. aureus* 2562 (L-MRSA) and *S. aureus* 2562 M2 (a methicillin-sensitive *S. aureus* (MSSA) subclone selected from *S. aureus* 2562 during subcultivation in drug-free media) was studied in an *in vitro* pharmacokinetic model simulating human plasma concentrations after an intravenous dose of 0.125 g to 2.0 g (Fig. 6). FK037 was highly bactericidal against H-MRSA only at doses of 1.0 g or higher. However, at doses of 0.125 g and 0.25 g, FK037 was bactericidal against MSSA and L-MRSA. On the other hand, flomoxef did not inhibit the re-growth of MSSA and L-MRSA even at 0.5 g and 1.0 g, respectively. In addition, flomoxef did not elicit bactericidal activity even at 2.0 g against H-MRSA.

Affinity of FK037 for PBPs of MRSA and Induction of PBP 2a of MRSA

The affinity of FK037 for PBPs of *S. aureus* 2562 M2 (MSSA) and PBP 2a of *S. aureus* 2562

Fig. 6. Bactericidal activity of FK037 and flomoxef against *Staphylococcus aureus* 2562 M2 (MSSA) (A), *S. aureus* 2562 (L-MRSA) (B) and *S. aureus* 2562 HR (H-MRSA) (C) in an *in vitro* pharmacokinetic model simulating human plasma concentrations after intravenous dosing.



L-MRSA, H-MRSA; See footnote in Table 1.

(MRSA) were compared with those of cefpirome and flomoxef. As shown in Table 4, FK037 inhibited PBPs 1, 2 and 3 of *S. aureus* 2562 M2 at I_{50} values of 0.58 μg/ml or lower, however, a higher concentration of 14 μg/ml was needed for PBP 4. The affinity of FK037 to MSSA-PBPs was similar to that of cefpirome and flomoxef, although the affinity of FK037 and cefpirome to PBP 4 was inferior to flomoxef. The I_{50} value of FK037 against PBP 2a of MRSA was 3.0 μg/ml, which is lower than that of cefpirome or flomoxef. As shown in Fig. 7, FK037 at 10 μg/ml inhibited 52% and 91% of non-treated PBP 2a within 30 and 120 minutes, respectively. This inhibition rate was faster than that of cefpirome and flomoxef. As shown in Fig. 8, FK037 at 0.1 and 1.0 μg/ml induced PBP 2a in *S. aureus* 302 (L-MRSA). Although the amount of PBP 2a induced by FK037 was lower than that by cefpirome, it was higher than that by flomoxef.

In Vitro Post-antibiotic Effect (*In Vitro* PAE) of FK037

PAEs of FK037 against H-MRSA and L-MRSA after 2-hour exposure are shown in Table 5. PAEs of FK037 against L-MRSA, strain 5039 at 12.5 and 25 μg/ml were 2.2 and 2.7 hours, respectively, whereas those against H-MRSA, strain 5027 at 25 to 100 μg/ml ranged from 1.2 to 1.7 hours. The PAE values were similar to those of cefpirome and exceeded those of flomoxef.

Therapeutic Effect of FK037 on Lethal Systemic Infection

The therapeutic effects of FK037 against 10 strains of MRSA were compared with those of the reference drugs (Table 6). The 10 strains, which comprised 2 L-MRSA and 8 H-MRSA strains, included 1 minocycline-resistant and 3 imipenem-resistant strains. The ED_{50} values of FK037 against the 10 strains ranged from 2.05 to 11.8 mg/kg. FK037 was active against the minocycline-resistant strain and the imipenem-resistant strains as well as the other MRSA strains. FK037 was significantly

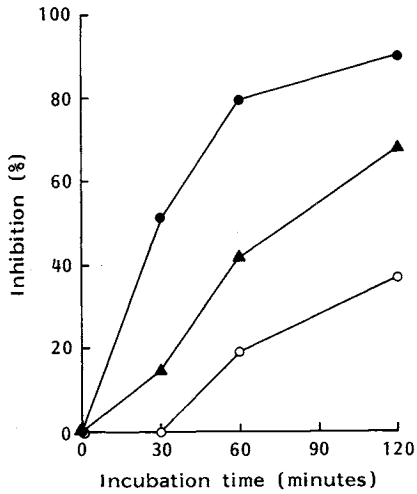
Table 4. Affinity of FK037 and reference drugs for penicillin-binding proteins of methicillin-sensitive *S. aureus* 2562 M2 and methicillin-resistant *S. aureus* 2562.

Organism	PBP	I_{50} (μg/ml)[a]		
		FK037	Cefpirome	Flomoxef
S. aureus 2562 M2	1	0.07	0.13	0.54
(*S. aureus* 2562)	2	0.06	0.14	0.12
	(2a)	3.0	5.4	52
	3	0.58	2.80	0.11
	4	14	>25	0.01

Abbreviation: PBP, penicillin-binding protein.
[a] Concentrations required to inhibit ^{14}C-benzylpenicillin binding to 50% of non-treated PBPs.

Fig. 7. Binding rate of FK037 and reference drugs to penicillin-binding protein 2a of methicillin-resistant *Staphylococcus aureus* 2562.

● FK037, ▲ cefpirome, ○ flomoxef.



more effective than cefpirome (against 7 of 8 strains) and flomoxef (against all the strains tested). Compared with imipenem, FK037 was more effective against 4 strains including 3 imipenem-resistant strains, equipotent against 4 strains but was less effective against 1 of 9 strains tested. Compared with vancomycin, FK037 was equipotent against 1 strain and was inferior against 4 of 5 strains tested. However, the ratios of the ED_{50} values of FK037 to those of vancomycin ranged from 1.2 to 3.4 and were markedly less than their MIC ratios, which ranged from 4 to 16. FK037 was less effective than minocycline against 2 minocycline-sensitive strains but was more effective than it against the minocycline-resistant strain.

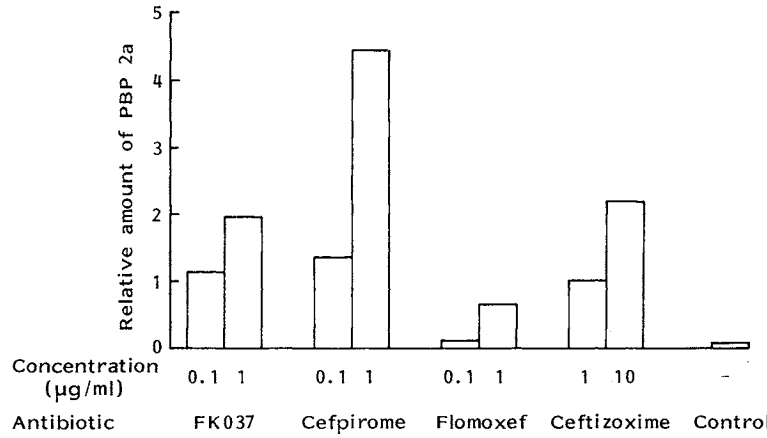
Influences of Challenge Dose on Therapeutic Effect of FK037

The therapeutic effects of FK037 on lethal systemic infections due to L-MRSA and H-MRSA strains at 2 challenge doses were compared with those of cefpirome and flomoxef (Table 7). At the lower challenge dose against *S. aureus* 5036 (L-MRSA), the therapeutic effect of FK037 was similar to that of cefpirome and flomoxef. However, at the higher challenge dose, the effect of FK037 was significantly more effective than the other 2 drugs. Against *S. aureus* 6004 (H-MRSA), FK037 was significantly more effective than the other 2 drugs at the lower challenge dose and the therapeutic effect ratio increased farther at the higher challenge dose.

Therapeutic Effect of FK037 on Pneumonia

The therapeutic effect of FK037 on pneumonia due to *S. aureus* 5027 (H-MRSA) was compared with that of the reference drugs (Fig. 9). FK037 significantly decreased the viable bacterial counts in the lungs to 4.04 $\log_{10}$ when compared with 7.91 $\log_{10}$ of the control mice. However, flomoxef (6.63 $\log_{10}$) and cefpirome (7.47 $\log_{10}$) did not exhibit the significant therapeutic effect although flomoxef had the same MIC as FK037.

Fig. 8. Induction of penicillin-binding protein 2a[a] of methicillin-resistant *Staphylococcus aureus* 302.



[a] Amount of PBP 2a induced by 1.0 μg/ml of ceftizoxime at 30°C for 6 hours was defined as 1.0.

Table 5. *In vitro* post-antibiotic effect of FK037 and reference drugs against methicillin-resistant *Staphylococcus aureus*.

Organism	Drug concentration (μg/ml)	*In vitro* PAE (hours)[a]		
		FK037	Cefpirome	Flomoxef
S. aureus 5039	25	2.7	3.0	1.8
(L-MRSA)[b]	12.5	2.2	2.3	1.3
S. aureus 5027	100	1.7	2.1	1.1
(H-MRSA)[b]	50	1.5	1.6	0.71
	25	1.2	1.0	0.45

[a] Bacteria were exposed to the drugs for 2 hours; [b] L-MRSA, H-MRSA: See footnote to Table 1.

Therapeutic Effect of FK037 on Endocarditis

FK037 significantly decreased the viable bacterial counts of *S. aureus* 5126 (H-MRSA) in the heart to 4.96 $\log_{10}$ when compared with 8.67 $\log_{10}$ of the control mice (Fig. 10). However, cefpirome (7.02 $\log_{10}$) and flomoxef (7.44 $\log_{10}$) did not exhibit the significant therapeutic effect. These findings correlated well with their MICs.

Therapeutic Effect of FK037 on Subcutaneous Abscess

Against a subcutaneous abscess due to *S. aureus* 9027 (H-MRSA), FK037 decreased the viable bacterial counts in the skin to 4.05 $\log_{10}$ when compared with 6.24 $\log_{10}$ of the control mice (Fig. 11). Contrarily, cefpirome (6.32 $\log_{10}$), flomoxef (6.74 $\log_{10}$) and imipenem (6.26 $\log_{10}$) had no effect compared to the control. Moreover, FK037 was as effective as vancomycin, although MIC value of FK037 was higher than that of vancomycin. Against a subcutaneous abscess due to *S. epidermidis* 9024 (MRSE), FK037 significantly decreased the viable bacterial counts in the skin to 4.20 $\log_{10}$ at 80 mg/kg, when compared with 6.70 $\log_{10}$ of the control mice (Fig. 12). However, at 80 mg/kg cefpirome (5.43 $\log_{10}$) had weak effect, and flomoxef (6.64 $\log_{10}$) and imipenem (6.43 $\log_{10}$) had no effect compared to control. The improved activity noted with FK037 compared with the other β-lactams may be reflective of the differences in the MICs against these organisms.

Table 6. Therapeutic effect of FK037 and reference drugs against lethal systemic infections with methicillin-resistant *Staphylococcus aureus* in mice.

Organism	Challenge[b] (cfu/mouse)	Antibiotic[d]	MIC[f] (μg/ml)	ED_{50} (95% confidence limit) (mg/kg)	
S. aureus 5011 (L-MRSA)[a]	1.7×10^8	FK037	3.13	2.05	(0.99~3.93)
		Cefpirome	3.13	4.86	(1.82~12.4)
		Flomoxef	1.56	6.61[g]	(4.56~10.9)
S. aureus 5092 (L-MRSA)[a] (Minocycline^R)	1.9×10^8	FK037	6.25	3.47	(1.67~6.80)
		Cefpirome	25	23.7[g]	(11.7~44.9)
		Flomoxef	1.56	8.48[g]	(4.33~16.6)
		Imipenem	0.1	0.74[h]	(0.37~1.40)
		Minocycline	25	>80.0	—
		Vancomycin	1.56	2.96	(1.46~5.61)
S. aureus 9098 (H-MRSA)[a]	1.6×10^8	FK037	6.25	4.92	(2.05~10.2)
		Cefpirome	25	19.7[g]	(8.22~40.8)
		Flomoxef	12.5	23.7[g]	(9.86~67.8)
		Imipenem	6.25	8.14	(3.15~19.2)
S. aureus 6004 (H-MRSA)[a]	1.6×10^8	FK037	12.5	7.76	(1.26~16.2)
		Imipenem	0.39	12.5	(0.006~26,400)
		Minocycline	0.78	0.869[h]	(0.42~1.70)
		Vancomycin	1.56	2.44[h]	(0.87~4.94)
S. aureus 9045 (H-MRSA)[a]	1.7×10^8	FK037	25	9.58	(4.86~19.2)
		Cefpirome	100	32.6[g]	(16.4~65.3)
		Flomoxef	100	47.8[g]	(23.2~123)
		Imipenem	6.25	8.31	(3.79~19.0)
		Vancomycin	1.56	3.49[h]	(1.80~6.74)
S. aureus 9087 (H-MRSA)[a]	1.7×10^8	FK037	12.5	11.8	(6.04~23.1)
		Cefpirome	25	47.2[g]	(24.2~92.5)
		Flomoxef	50	192[g]	(97.6~480)
		Imipenem	12.5	30.3[i]	(13.8~113)
S. aureus 5027 (H-MRSA)[a]	2.1×10^8	FK037	12.5	11.8	(5.86~22.4)
		Imipenem	1.56	10.6	(3.70~25.4)
		Minocycline	0.39	0.298[h]	(0.14~0.61)
		Vancomycin	0.78	3.47[h]	(1.67~6.80)
S. aureus 10003 (H-MRSA)[a] (Imipenem^R)	1.9×10^8	FK037	25	6.85	(2.78~15.3)
		Cefpirome	100	60.5[g]	(36.7~87.7)
		Flomoxef	100	>160	—
		Imipenem	50	77.9[g]	(37.4~4,640)
S. aureus 10004 (H-MRSA)[a] (Imipenem^R)	1.6×10^8	FK037	25	8.43	(4.50~15.9)
		Cefpirome	100	23.6[g]	(12.1~46.2)
		Flomoxef	100	67.6[g]	(35.6~137)
		Imipenem	50	33.8[g]	(17.8~68.3)
S. aureus 9002 (H-MRSA)[a] (Imipenem^R)	1.6×10^7[c]	FK037[e]	25	9.98	(8.14~12.2)
		Cefpriome[e]	50	40.2[g]	(32.7~49.3)
		Flomoxef[e]	100	80.1[g]	(42.9~152)
		Imipenem[e]	50	26.1[g]	(13.8~38.6)
		Vancomycin[e]	1.56	3.00[g]	(1.53~7.50)

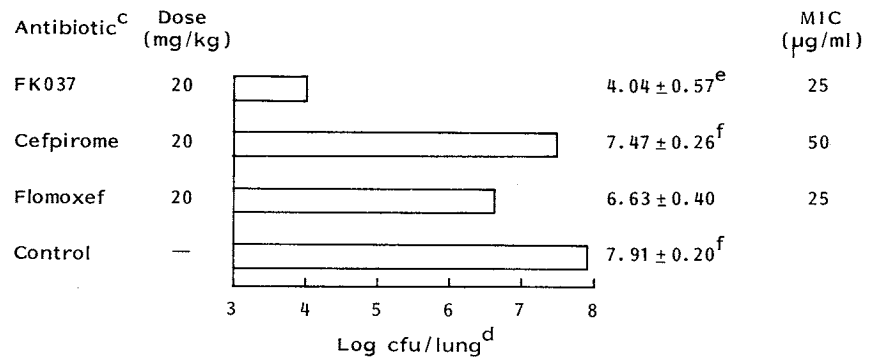
[a] See footnote to Table 1; [b] mice were intraperitoneally inoculated with 0.5 ml of bacterial suspension in 5% gastric mucin; [c] mice were immunosuppressed with intraperitoneal cyclophosphamide at 200 mg/kg 4 days before inoculation of bacterial suspension; [d] antibiotic was subcutaneously given in mice 1 hour after inoculation of bacteria; [e] antibiotic was subcutaneously given in mice 1 and 3 hours after inoculation; [f] agar dilution method (stamp method, 10^4 cfu/spot), Mueller-Hinton agar; [g] statistical significances refer to parallel line assay for paired differences, significant difference ($P<0.05$), FK037 > reference antibiotic, [h] FK037 < reference antibiotic, [i] significant difference ($P<0.10$), FK037 > reference antibiotic.

Table 7. Influence of challenge dose on therapeutic effect of FK037 and reference drugs on lethal systemic infection with methicillin-resistant *Staphylococcus aureus* in mice.

Organism	Challenge dose[b] (cfu/mouse)	ED_{50} (mg/kg)[d]		
		FK037	Cefpirome	Flomoxef
S. aureus 5036 (L-MRSA)[a]	1.6×10^8 (L)	2.50	2.04	2.50
	5.3×10^8 (H)	3.29	10.0[e]	10.0[e]
	ED_{50} Ratio (H/L)	1.3	4.9	4.0
	MIC (μg/ml)[c]	1.56	0.78	0.78
S. aureus 6004 (H-MRSA)[a]	1.6×10^8 (L)	8.20	23.7[e]	23.5[e]
	2.5×10^8 (H)	19.3	233[e]	276[e]
	ED_{50} Ratio (H/L)	2.4	9.8	11.7
	MIC (μg/ml)[c]	12.5	25	25

[a] See footnote to Table 1; [b] mice were intraperitoneally inoculated with 0.5 ml of bacterial suspension in 5% gastric mucin; [c] agar dilution method (stamp method; 10^4 cfu/spot), Mueller-Hinton agar; [d] antibiotic was given subcutaneously 1 hour after inoculation of bacteria; [e] statistical significances refer to parallel line assay for paired differences, significant difference ($P<0.05$), FK037 > reference drug.

Fig. 9. Therapeutic effect of FK037 and reference drugs on pneumonia with highly methicillin-resistant *Staphylococcus aureus* 5027[a] in mice[b].



[a] Bacterial suspension (0.05 ml) of *S. aureus* 5027 (1.8×10^6 cfu) in saline were intranasally inoculated in the anesthetized mice; [b] mice were immunosuppressed by intraperitoneal cyclophosphamide at 200 mg/kg 4 days before infection; [c] mice were subcutaneously treated with the antibiotics at 20 mg/kg/administration for 6 times (post-infection 3, 6, 24, 32, 48 and 56 hours); [d] observation, 3 days after infection; [e] statistical significances refer to Dunnett-type multiple comparison for paired differences, significant difference from control ($P<0.05$); [f] significant difference from FK037 ($P<0.05$).

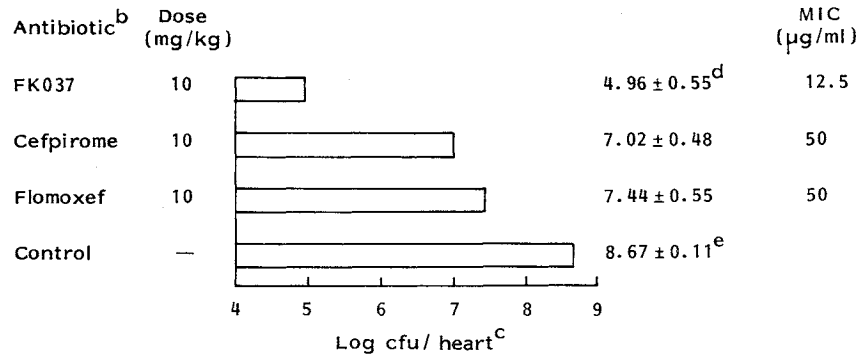
Therapeutic Effect of FK037 on Intrauterine Infection

FK037 significantly decreased the viable bacterial counts of *S. aureus* 5027 (H-MRSA) in the uterus to 3.70 $\log_{10}$ at 80 mg/kg when compared with 7.01 $\log_{10}$ of the control mice (Fig. 13). However, at 80 mg/kg flomoxef (5.38 $\log_{10}$) and cefpirome (4.94 $\log_{10}$) did not exhibit the significant therapeutic effect, although flomoxef had the same MIC as FK037.

Therapeutic Effect of FK037 on Granuloma Pouch Infection

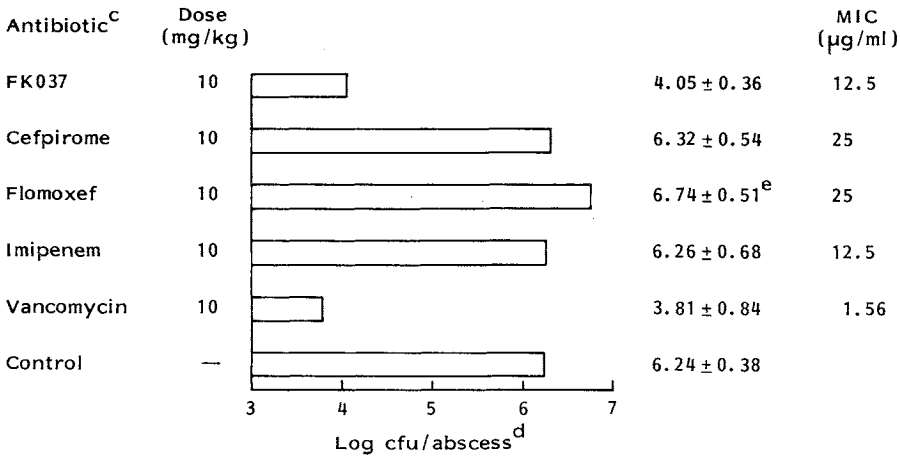
In granuloma pouch infection due to *S. aureus* 5027 (H-MRSA), FK037 at 80 mg/kg was significantly bactericidal in the exudate fluid 2 and 3 days after infection when compared with those of the control mice (Fig. 14). Neither cefpirome nor flomoxef significantly decreased the viable bacterial counts at the

Fig. 10. Therapeutic effect of FK037 and reference drugs on endocarditis with highly methicillin-resistant *Staphylococcus aureus* 5126[a] in rats.



[a] Challenge, 5.0×10^4 cfu, iv, 24 hours after cannulation into the heart; [b] rats were intravenously treated with 10 mg/kg/administration of the antibiotic for 3 administration at post-infection 5, 24 and 32 hours; [c] observation, 2 days after infection; [d] statistical significances refer to Dunnett-type multiple comparison for paired differences, significant difference from control ($P<0.05$); [e] significant difference from FK037 ($P<0.05$).

Fig. 11. Therapeutic effect of FK037 and reference drugs on subcutaneous abscess with highly methicillin-resistant *Staphylococcus aureus* 9027[a] in mice[b].



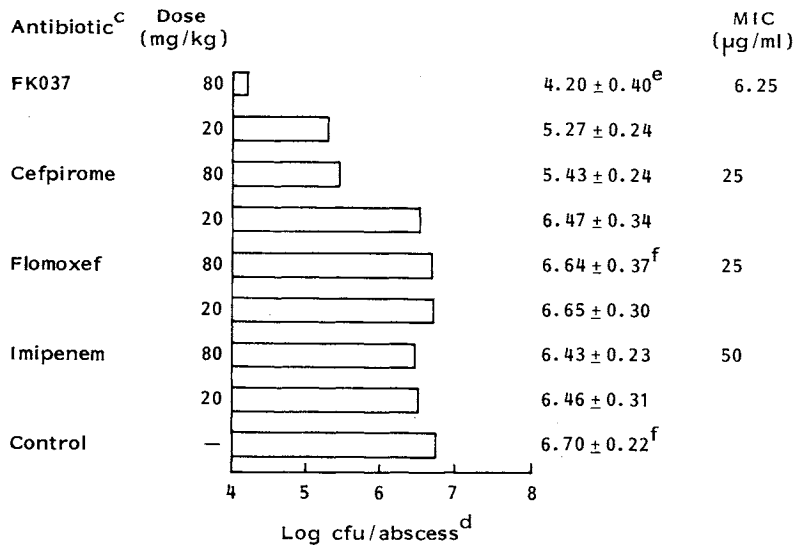
[a] Challenge, 1.2×10^3 cfu, sc; [b] mice were immunosuppressed with intraperitoneal cyclophosphamide at 200 mg/kg 4 days before infection; [c] mice were subcutaneously treated with 10 mg/kg/administration of antibiotics for 5 administrations at post-infection 5, 24, 32, 48 and 56 hours at sites remote from the infection sites; [d] observation, 3 days after infection; [e] statistical significances refer to Dunnett-type multiple comparison for paired difference, significant difference from FK037 ($P<0.05$).

80 mg/kg dose.

In Vivo Frequency of Highly Resistant Cells to Methicillin

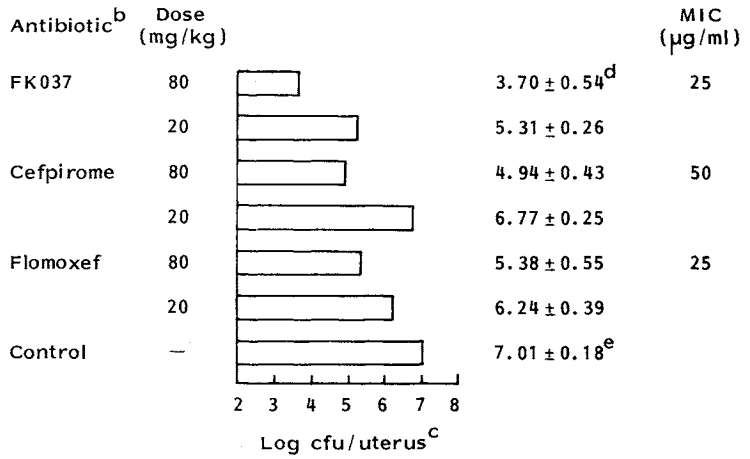
The frequency of highly methicillin-resistant cells in the exudate fluid after repeated dosing of 20 mg/kg FK037 in a granuloma pouch infection due to *S. aureus* 5027 (H-MRSA) was compared with that after repeated dosing of the reference drugs at 20 mg/kg (Fig. 15). The *in vivo* frequency of the cells highly resistant to methicillin by FK037 treatment ($10^{-2.97}$) was nearly equal to that in non-treated control ($10^{-3.18}$), and was significantly lower than that with cefpirome treatment ($10^{-2.24}$) and flomoxef

Fig. 12. Therapeutic effect of FK037 and reference drugs on subcutaneous abscess with highly methicillin-resistant *Staphylococcus epidermidis* 9024[a] in mice[b].



[a] Challenge, 4.4×10^6 cfu, sc; [b] mice were immunosuppressed with intraperitoneal cyclophosphamide at 200 mg/kg 4 days before infection; [c] mice were subcutaneously treated with 80 and 20 mg/kg/administration of antibiotics for 5 administrations at post-infection 5, 24, 32, 48 and 56 hours at sites remote from the infection sites; [d] observation, 3 days after infection; [e] statistical significances refer to Tukey-type multiple comparison for paired difference, significant difference from control ($P<0.05$); [f] significant difference from FK037 ($P<0.05$).

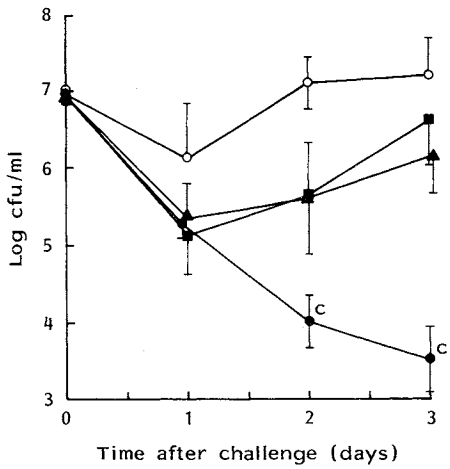
Fig. 13. Therapeutic effect of FK037 and reference drugs on intrauterine infection with highly methicillin-resistant *Staphylococcus aureus* 5027[a] in mice.



[a] Challenge, 5.5×10^5 cfu, left uterine horn; [b] mice were subcutaneously treated with 80 and 20 mg/kg/administration of antibiotics for 5 administrations at post-infection 5, 25, 32, 48 and 56 hours; [c] observation, 3 days after infection; [d] statistical significances refer to Tukey-type multiple comparison for paired differences, significant difference from control ($P<0.05$); [e] significant difference from FK037 ($P<0.05$).

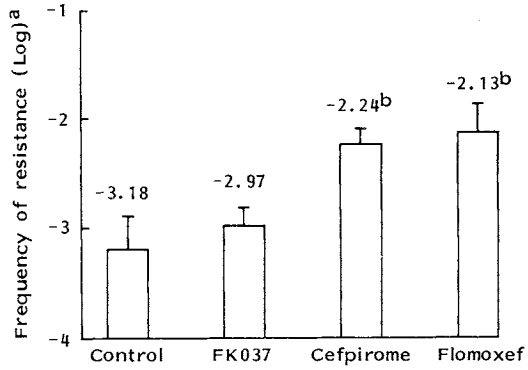
Fig. 14. Therapeutic effect of FK037 and reference drugs (80 mg/kg) on granuloma pouch infection with highly methicillin-resistant *Staphylococcus aureus* 5027[a] in rats.

○ Control, ● FK037[b], ▲ cefpirome[b], ■ flomoxef[b].



[a] Challenge, 4.4×10^7 cfu in 5% gastric mucin, into granuloma pouch; [b] rats were intramuscularly treated with antibiotics at post-infection 5, 24, 32, 48 and 56 hours; [c] statistical significances refer to Dunnett-type multiple comparison for paired differences, significance difference from control ($P<0.05$).

Fig. 15. *In vivo* frequency of highly methicillin-resistant cells in the exudate fluid after 5 repeated dosing (20 mg/kg) of FK037 and reference drugs in granuloma pouch infection with highly methicillin-resistant *Staphylococcus aureus* 5027 in rats.



[a] Ratio of the number of colonies on methicillin-containing (200 μg/ml) plate to that on control plate; [b] statistical significances refer to Dunnett-type multiple comparison for paired differences, significant difference from FK037 ($P<0.05$).

treatment ($10^{-2.13}$). These results indicate that repeated treatment with FK037 did not produce selection of highly methicillin-resistant cells, although repeated treatment with cefpirome and flomoxef did.

Therapeutic Effect of FK037 on Pneumonia in an *In Vivo* Pharmacokinetic Model

The therapeutic effect of FK037, cefpirome and flomoxef on pneumonia due to *S. aureus* 5027 (H-MRSA) was compared at doses equivalent to 1.0 and 2.0 g in humans (Fig. 16). The viable counts in the lungs from FK037-treated mice at both doses were significantly lower than those from control mice 6 and 24 hours after the initiation of dosing. The therapeutic effects with the 2.0 g dose were more potent than that with 1.0 g dose. However, the viable counts in the lungs from cefpirome-treated mice were significantly lower than those from control mice only at 2.0 g, and those from flomoxef-treated mice were not significantly different from those from control mice even at 2.0 g, indicating that the therapeutic effects of FK037 against H-MRSA pneumonia were stronger than those of cefpirome and flomoxef.

In Vivo Post-antibiotic Effect (*In Vivo* PAE) of FK037

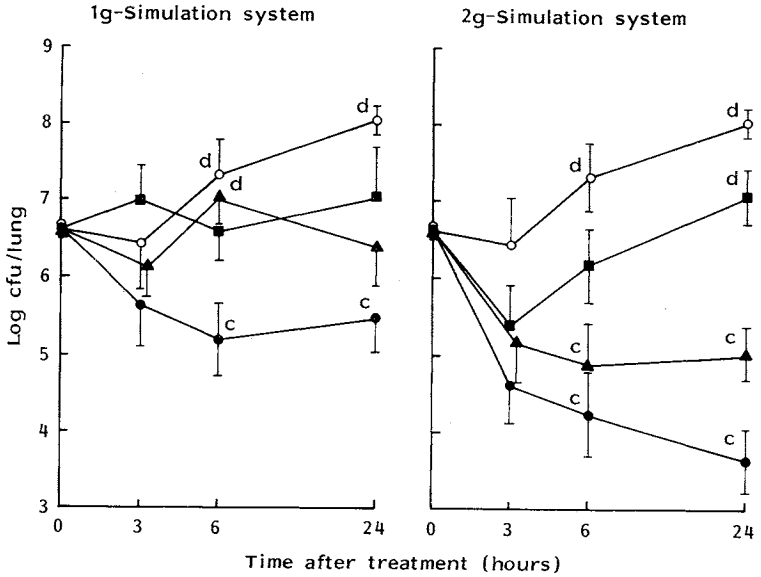
As shown in Fig. 17, FK037 produced *in vivo* PAE of 4.1 hours against a thigh infection with *S. aureus* 5027 (H-MRSA) in neutropenic mice.

Synergistic Therapeutic Effect of FK037 with either Imipenem or Fosfomycin on Lethal Systemic Infections

The therapeutic effects of FK037 in combination with either imipenem or fosfomycin against a lethal systemic infection due to *S. aureus* 5027 (H-MRSA) or *S. aureus* 6004 (H-MRSA) were compared with those of each drug alone (Table 8). Considering their usual clinical doses, a combination ratio of 4 : 1 or 1 : 1 was used for FK037 with either imipenem or fosfomycin, respectively. FED indexes by FK037/imipenem

Fig. 16. Therapeutic effect of FK037 and reference drugs on murine pneumonia[a] in an *in vivo* pharmacokinetic model simulating human plasma concentrations after drip infusion of 1 g and 2 g.

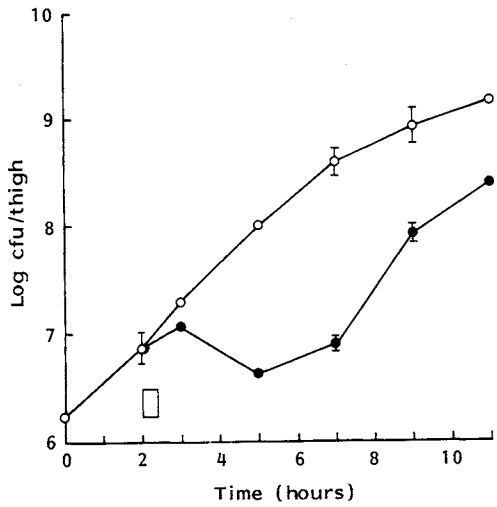
○ Control, ● FK037[b], ▲ cefpirome[b], ■ flomoxef[b].



[a] Mice were immunosuppressed with 200 mg/kg cyclophosphamide (ip) 4 days before infection. Volumes of 0.05 ml of *S. aureus* 5027 (2.6×10^6 cfu) in saline were intranasally inoculated in anesthetized mice; [b] mice were treated with antibiotics 16 hours after infection; [c] statistical significances refer to Dunnett-type multiple comparison for paired differences, significant difference from control ($P<0.05$); [d] significant difference ($P<0.05$) from FK037.

Fig. 17. *In vivo* post-antibiotic effect of FK037 against a thigh infection with highly methicillin-resistant *Staphylococcus aureus* 5027 in neutropenic mice.

○ Control, ● FK 037, □ time thigh levels exceeded the MIC.



combination against 2 strains were 0.567 and 0.358, and their ED_{50} values with concomitant administration were lower than those when the drugs were used alone. The FK037/fosfomycin combination was synergistic only against fosfomycin-sensitive *S. aureus* 6004.

Discussion

A major advantage of FK037 over the commercially available cephalosporins, cephalosporins in development and imipenem is its potent *in vitro* and *in vivo* activities against methicillin-resistant staphylococci[1,2]. In the present study, the potent *in vitro* activities of FK037 against MRSA and MRCNS relative to those of the reference drugs were confirmed (Table 1). In particular, against H-MRSA and H-MRCNS, FK037 was the most active agent with no highly resistant strains with MICs of $>100\,\mu g/ml$ (Fig. 1). The potent *in vitro*

Table 8. *In vivo* combination effects of FK037 with either imipenem or fosfomycin on lethal systemic infection with highly methicillin-resistant *Staphylococcus aureus*[a] in mice.

Organism	Antibiotic (combination ratio)[b]	ED_{50} (mg/kg)	FED index	MIC (μg/ml)
S. aureus 5027 (2.4×10^8 cfu, ip)	FK037 alone	16.4		12.5
	Imipenem alone	40.0		1.56
	FK037/imipenem combination (4:1)	8.43/2.11	0.567	
	FK037 alone	16.4		12.5
	Fosfomycin alone	>320		≧200
	FK037/fosfomycin combination (1:1)	16.3/16.3	0.997	
S. aureus 6004 (2.8×10^8 cfu, ip)	KF037 alone	16.3		12.5
	Imipenem alone	24.4		0.39
	FK037/imipenem combination (4:1)	5.0/1.25	0.358	
S. aureus 6004 (2.2×10^8 cfu, ip)	FK037 alone	27.9		12.5
	Fosfomycin alone	5.0		12.5
	FK037/fosfomycin combination (1:1)	1.61/1.61	0.380	

[a] See footnote to Table 1; [b] antibiotic was given subcutaneously 1 hour after infection.

activity of FK037 against MRSA may be due to the high affinity (Table 4) and binding rate to PBP 2a (Fig. 7). The same reason can be applied for the limited influence which some culture conditions, such as low temperature and supplementation with 4% NaCl which increase PBP 2a activity and high inoculum have on the anti-MRSA activity of FK037 (Fig. 2). This is particularly clear when compared with the cases of cefpirome, flomoxef and imipenem. β-Lactam-resistance in MRSA is usually heterogeneous and most MRSA contains only a limited number of highly resistant cells[20]. However, FK037 had an advantage in that the *in vitro* incidence of highly resistant strains in MRSA was lower with FK037 than with cefpirome or flomoxef (Fig. 4). A slower development of *in vitro* resistance occurred with FK037 than with cefpirome or flomoxef (Fig. 5). Also no colonies occurred inside the inhibition zone of FK037, although colonial growth inside the inhibition zone of flomoxef and imipenem was evident (Fig. 3). In addition, the *in vivo* incidence of cells highly resistant to methicillin after repeated dosing of FK037 in a granuloma pouch infection due to H-MRSA was lower than that obtained with cefpirome and flomoxef (Fig. 16). These findings suggest that the possibility of selection of such resistant cells will be low with FK037 therapy in humans.

Against most of the lethal systemic infections with MRSA, including imipenem-resistant and minocycline-resistant strains, FK037 had greater therapeutic activity than cefpirome or flomoxef and was either equipotent or more effective than imipenem (Table 6). The maximum FK037 ED_{50} obtained in the present study was 11.8 mg/kg, which is markedly lower than that of 60.5 mg/kg for cefpirome, 192 mg/kg for flomoxef and 77.9 mg/kg for imipenem. This may be related to the more potent *in vitro* activity of FK037 compared with the reference drugs against H-MRSA. However, the *in vivo* activity of FK037 against MRSA was more potent than had been anticipated from its *in vitro* activity compared with those of the reference drugs. In addition, although the therapeutic effect of FK037 was weaker than that of vancomycin, the ratio of the ED_{50} values of FK037 to that of vancomycin were lower (ranging from 1.2 to 3.4), suggesting that FK037 is as effective as vancomycin because its usual clinical dose is probably 4 times that of vancomycin. Moreover, compared with the reference drugs, only a limited influence of the challenge dose on the therapeutic effect was noted with FK037. This result coincided with the limited influence on the *in vitro* anti-MRSA activity noted by changes in the inoculum level (Table 7). In addition to its activity against lethal systemic infections, FK037 displayed potent therapeutic effects in the following experimental local infection models simulating various common human infections due to MRSA and MRSE: Pneumonia (Fig. 9), endocarditis (Fig. 10), subcutaneous abscess (Fig. 11 and 12), intrauterine infection (Fig. 13) and granuloma pouch infection (Fig. 14). FK037 was the most effective of the β-lactams tested. In addition, the equipotential therapeutic effect of FK037 to vancomycin against subcutaneous abscess is noteworthy since vancomycin is currently the best available drug against MRSA infections.

Moreover, against H-MRSA pneumonia in an *in vivo* pharmacokinetic model simulating human plasma concentrations, the viable counts in the lungs from FK037 treated animals at doses equivalent to 1.0 and 2.0 g in human were lower than those from cefpirome treated and flomoxef treated animals (Fig. 16). These results strongly suggest that FK037 is useful in treating various MRSA and MRSE infections in humans. In addition to the potent *in vitro* and *in vivo* anti-MRSA activity of FK037 alone, synergistic responses were obtained with combinations of FK037 with either imipenem or fosfomycin against MRSA suggesting that such concomitant administrations may be useful for treating severe MRSA infections when FK037 alone was found to be inadequate. In conclusion, FK037 displays a major beneficial advantage against MRSA over cefpirome, flomoxef and imipenem.

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