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In Vitro Antibacterial Activity of FK041, a New Orally Active Cephalosporin

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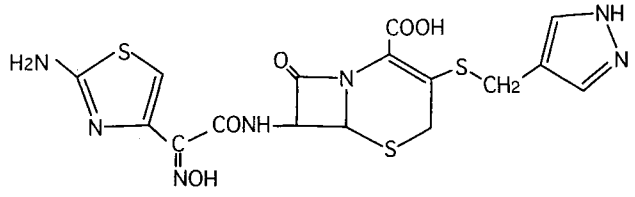
The *in vitro* activity of FK041, a new orally active cephem antibiotic, against a wide variety of clinical isolates of bacteria was investigated and compared with those of cefdinir (CFDN) and cefditoren (CDTR). FK041 exhibited broad spectrum activity against reference strains of Gram-positive and Gram-negative aerobes and anaerobes. FK041 was active against clinical isolates of Gram-positive organisms except *Enterococcus faecalis* with MIC_{90}s less than 1.56 μg/ml. FK041 was more active than CFDN and CDTR against *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Streptococcus agalactiae* and was comparable to CFDN and CDTR against *Streptococcus pyogenes* and *Streptococcus pneumoniae*. FK041 had no activity against methicillin-resistant staphylococci, like CFDN and CDTR. FK041 showed moderate activity against penicillin-resistant *S. pneumoniae* with an MIC range of 0.05~3.13 μg/ml , and was superior to CFDN but inferior to CDTR. Against clinical isolates of many Gram-negative organisms such as *Neisseria gonorrhoeae*, *Escherichia coli*, *Klebsiella pneumoniae*, and *Proteus mirabilis*, FK041 had good activity comparable or superior to those of CFDN and CDTR. However, it was inferior to CDTR in activity against *Moraxella catarrhalis*, *Haemophilus influenzae*, *Morganella morganii*, and *Serratia marcescens*, and was inactive against *Pseudomonas aeruginosa*. With FK041 a small difference between MIC and MBC against *S. aureus*, *E. coli*, *K. pneumoniae*, and *H. influenzae* was found, indicating that its action is bactericidal against these species. FK041 was stable to group 2 β-lactamase hydrolysis but was unstable to group 1 β-lactamase hydrolysis. The stability of FK041 to these enzymes was similar to those of CFDN and CDTR. FK041 showed high affinity for the main penicillin-binding proteins (PBPs) of *S. aureus* (PBP 3, 2, and 1) and *E. coli* (PBP 3, 4, 1bs, 2, and 1a).

In the past 30 years, various orally active cephem antibiotics have been developed with the aim of improving antibacterial potency and oral absorbability[1~3]. The clinical usefulness of oral α-aminocephems was limited, however, because of their relatively low activity against Gram-negative bacilli. In order to overcome this defect, new oral cephalosporins such as cefixime (CFIX)[4] and cefteram[5] with potent activity against Gram-negative bacteria and good stability to β-lactamase hydrolysis from Gram-negative bacteria were developed in the early 1980s. Although these compounds had advantages in their spectrum of activity against Gram-negative bacteria, the activity against staphylococci was not satisfactory. In 1988, we reported cefdinir (CFDN) with potent activity against Gram-positive bacteria, while maintaining the antibacterial spectrum of CFIX against Gram-negative strains[6~8].

We recently found a new orally active cephalosporin, FK041, in the course of screening for more potent activity and higher oral absorption[9,10]. In order to define clearly the antibacterial activity of FK041, a comprehensive *in vitro* evaluation of antibacterial activity was undertaken.

Fig. 1. Chemical structure of FK041.



(6*R*,7*R*)-7-[(*Z*)-2-(2-Amino-4-thiazolyl)-2-hydroxyiminoacetamido]-8-oxo-3-(1*H*-pyrazol-4-yl) methylthio-5-thia-1-azabicyclo [4.2.0.]oct-2-ene-2-carboxylic acid.

Materials and Methods

Antibiotics

FK041, CFDN, and cefditoren (CDTR) were synthesized in the Medicinal Chemistry Research Laboratories, Fujisawa Pharm. Co., Ltd., Osaka, Japan.

Bacterial Strains

Reference strains from the culture collection in our laboratories were used in this study. Clinical isolates of various species of bacteria were obtained from several hospitals in Japan. The criteria for drug resistance were MICs of $\geqq$12.5 μg/ml of methicillin for methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-resistant *Staphylococcus epidermidis* (MRSE); $\geqq$0.1 μg/ml of benzylpenicillin for penicillin G-resistant *Streptococcus pneumoniae* (PRSP); $\geqq$3.13 μg/ml of benzylpenicillin for penicillin G-resistant *Haemophilus influenzae*; $\geqq$3.13 μg/ml of levofloxacin (LVFX) for fluoroquinolone-resistant *Escherichia coli, Klebsiella pneumoniae*, and *Proteus mirabilis*.

Antibiotic Susceptibility Testing

MICs were determined by the agar dilution method according to the National Committee for Clinical Laboratory Standards (NCCLS) reference method[11,12] with slight modification. Briefly, Mueller-Hinton agar (Difco Laboratories, Detroit, USA) was used for nonfastidious aerobic bacteria. This medium, supplemented with 5% defibrinated horse blood was used for *Streptococcus pyogenes*, *S. agalactiae*, *S. pneumoniae*, *S. mitis*, *S. mutans*, *Corynebacterium diphtheriae*, and *Bordetella pertussis* and with heated 5% defibrinated horse blood (chocolate agar) for *Neisseria* species and *H. influenzae*, respectively. Modified-GAM agar (Nissui, Tokyo, Japan) was used for testing the anaerobic bacteria. The nonfastidious aerobic organisms were precultured in Mueller-Hinton broth (Difco); *Staphylococcus* species, *Micrococcus luteus*, *Enterococci* species and *Moraxella catarrhalis* were precultured in Trypticase soy broth (BBL Microbiology System, Cockeysville, USA); *S. pneumoniae*, *S. pyogenes*, *S. agalactiae*, *S. mitis S. mutans C. diphtheriae* and *B. pertussis* were precultured on Trypticase soy agar with 5% defibrinated horse blood; *Neisseria* species and *H. influenzae* were precultured on Mueller-Hinton agar with 5% defibrinated horse blood (chocolate agar); and anaerobic bacteria were precultured in GAM broth (Nissui). Overnight cultures of these organisms were diluted 100-fold with the respective medium, and an inoculum containing 10^4 cfu was applied with a multipoint replicating apparatus to agar plates containing serial 2-fold dilutions of each antibiotic. These plates were incubated at 37°C for 18 or 48 hours. For streptococci except *S. pyogenes, H. influenzae, Neisseria* species, *C. diphtheriae*, and *B. pertussis*, incubation was carried out in an atmosphere of 5% CO_2, and for anaerobic bacteria, incubation was carried out in an anaerobic system Model 1024 (Forma Scientific, Inc. OH, USA) at 37°C for 24 or 48 hours. The MIC was the lowest antibiotic concentration that inhibited macroscopic colonial growth during this incubation.

Determination of Minimum Bactericidal Concentrations (MBC)

MBC values were determined by the microdilution broth method in Mueller-Hinton broth according to the NCCLS reference method[11]. For *H. influenzae*, the medium was supplemented with 5% Fildes enrichment (Oxoid, Ltd. Hampshire, UK). The drugs dissolved in the medium at 100 μg/ml were serially diluted 2-fold and 100 μl of each broth was dispersed into 96-well multiwell trays with a dispenser (MIC 2000 system; Dynatech Laboratories, Inc., VA, USA). The strains precultured on Mueller-Hinton agar (chocolate agar for *H. influenzae*) at 37°C for 18 hours were suspended in saline for turbidity adjustment to McFarland 0.5 standard. About 1 μl samples of the suspension were inoculated into broths containing compounds with an automatic inoculator (MIC 2000 system; Dynatech) to achieve a final concentration at about 10^6 cfu/ml. After incubation at 37°C for 18 hours, the MIC was read as the lowest drug concentration that inhibited visible bacterial growth. About 1 μl of the culture broth in each well was inoculated onto Mueller-Hinton agar (chocolate agar for *H. influenzae*) with an automatic inoculator. After incubation at 37°C for 18 hours, the MBC which killed 99.9% of inoculated bacteria was read as the lowest compound concentration that inhibited colony formation. Incubation was carried out in an atmosphere of 5% CO_2

for *H. influenzae*.

β-Lactamase Assay

After disruption by sonication, β-lactamases from several strains were purified by gel filtration *via* a Sephadex G-100 column. Enzymatic hydrolysis rates of compounds were determined with spectrophotometer at 37°C in a 67 mM phosphate buffer (pH 7.0). The rate of hydrolysis of the β-lactam ring was followed at 37°C by the change in absorption at 294 nm for FK041, 310 nm for CFDN and

Table 1. Antibacterial spectrum of FK041 against aerobic organisms.

Organism	MIC (μg/ml)		
	FK041	CFDN	CDTR
Staphylococcus aureus 209P JC-1	0.1	0.1	0.39
S. aureus 5027 (MRSA)	50	>100	100
S. epidermidis ATCC 14990	0.05	0.05	0.05
Micrococcus luteus ATCC 9341	0.05	0.05	≦0.025
Streptococcus pyogenes ATCC 10389[b]	0.1	≦0.025	≦0.025
S. agalactiae[b, d]	0.05	0.05	0.05
S. mitis ATCC 9811[b, d]	0.2	0.2	0.05
S. mutans ATCC 10449[b, d]	0.2	0.2	0.05
S. pneumoniae III[b, d]	≦0.025	0.05	≦0.025
Enterococcus faecalis ATCC 29212	3.13	6.25	100
E. faecium ATCC 19434	3.13	25	100
E. avium ATCC 14025	0.78	3.13	1.56
Corynebacterium diphtheriae NIHJ A-7[b, d]	0.1	0.2	0.39
Bacillus subtilis ATCC 6633	0.2	0.78	0.39
Neisseria gonorrhoeae ATCC 49226[c, d]	≦0.025	≦0.025	≦0.025
N. meningitidis[c, d]	≦0.025	≦0.025	≦0.025
Moraxella catarrhalis 8005	≦0.025	0.2	≦0.025
Bordetella pertussis[a, b, d]	3.13	12.5	0.39
Escherichia coli NIHJ JC-2	0.05	0.2	0.1
Citrobacter freundii N0.2	0.2	0.39	0.78
Salmonella typhi T-287	0.05	0.2	0.1
S. typhimurium 1406	≦0.025	0.1	0.2
S. enteritidis 116-54	0.05	0.2	0.78
Shigella dysenteriae A1 shiga	0.1	0.39	0.2
S. sonnei I EW 33	≦0.025	0.2	0.2
Klebsiella pneumoniae IFO 3512	≦0.025	0.05	≦0.025
K. oxytoca ATCC 13182	≦0.025	0.05	0.05
Enterobacter cloacae ATCC 13047	12.5	50	6.25
E. aerogenes ATCC 13048	0.39	1.56	0.78
Serratia marcescens IFO 3736	1.56	6.25	0.78
Proteus mirabilis IFO 3849	0.05	0.1	0.2
P. vulgaris IFO 3858	≦0.025	0.05	≦0.025
Morganella morganii IFO 3848	≦0.025	0.1	≦0.025
Providencia rettgeri IFO13501	≦0.025	≦0.025	≦0.025
P. stuartii No.64	0.05	0.1	0.39
Pseudomonas aeruginosa ATCC 27853	>100	>100	25
P. putida KM624	1.56	6.25	0.78
Burkholderia cepacia ATCC 25416	25	50	25
Stenotrophomonas maltophilia ATCC 13637	>100	>100	100
Chryseobacterium meningosepticum ATCC 13253	50	100	12.5
Haemophilus influenzae ATCC 9334[c, d]	0.2	0.39	0.05
Acinetobacter calcoaceticus ATCC 15309	≦0.025	0.05	0.2
Alcaligenes faecalis ATCC 15554	0.39	1.56	6.25

Agar dilution method (stamp method): Mueller-Hinton agar, 10^4 cfu/spot, 37°C, 18 or [a]48 hours; [b]supplemented with 5% defibrinated horse blood ([c]chocolate agar), [d]5% CO_2.

305 nm for CDTR. *Km* and Vmax values were obtained by a least-squares fit of the initial steady-state velocities at different substrate concentrations. *Ki* values were alternatively determined for the enzyme-stable compounds by measuring the inhibition of enzymatic degradation of Nitrocephin. Relative Vmax and relative Vmax/*Km* values of FK041 and the reference compounds were expressed as the percentage of those of cephaloridine for group 1 β-lactamase and those of ampicillin for group 2 β-lactamase.

Assay of Affinity for Penicillin-binding Proteins (PBPs)

The affinity of the test antibiotic for the PBPs of *S. aureus* 209P JC-1, *E. coli* NIHJ JC-2, and *H. influenzae* 7018 was assayed by a modification of Spratt's method, as described previously[13]. Membrane fractions containing PBPs were prepared from bacteria after sonication with a sonicator Model 200M (Kubota, Tokyo, Japan) and ultracentrifugation at 100,000×*g*. For preparation of membrane fractions from *S. aureus*, lysostaphin (Sigma Chemical Co., St. Louis, MO, USA) treatment at 50 μg/ml at 30°C for 30 minutes was performed before sonication. The membrane fractions were exposed to compounds at 30°C for 10 minutes. ^{14}C-labeled benzylpenicillin (^{14}C-PCG, specific activity at 58.5 mCi/mmol, Amersham) was used for detection of unsaturated PBPs by pretreatment with drugs. The labeled PBPs were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 10% acrylamide and 0.12% bisacrylamide. The dried gels were applied to BAS2000 System (Fuji Film, Tokyo, Japan) and the of drugs which inhibited the ^{14}C-PCG binding to 50% of non-treated PBPs (IC_{50}) were determined as the affinity of drugs to PBPs.

Results

Antibacterial Spectrum of FK041

The activities of FK041 against 43 reference strains of aerobic bacteria are shown in Table 1. FK041 had potent antibacterial activity against an extensive range of organisms. FK041 was more active than CFDN against most Gram-positive and Gram-negative bacteria. FK041 exhibited more potent activity than CDTR against *S. aureus* and enterococci, however, it was less active against *S. pyogenes S. mitis* and *S. mutans*. The activity of FK041 was similar or superior to that of CDTR against most Gram-negative bacteria, except that it was less active

Table 2. Antibacterial spectrum of FK041 against anaerobic organisms.

Organism	MIC (μg/ml)		
	FK041	CFDN	CDTR
Peptostreptococcus magnus ATCC 14955	0.05	0.2	1.56
P. asaccharolyticus ATCC 14963	0.2	0.78	0.78
P. prevotii ATCC 9321	0.39	1.56	0.39
Propionibacterium acnes ATCC 11827	≦0.025	≦0.025	≦0.025
Bifidobacterium adolescentis ATCC 15703	0.1	0.39	0.2
Eubacterium lentum ATCC 25559	3.13	6.25	3.13
Clostridium perfringens ATCC 3624	0.78	0.39	≦0.025
C. tetani ATCC 10779	0.1	0.39	0.1
C. difficile ATCC 9689	12.5	25	25
Lactobacillus casei ATCC 393	1.56	1.56	0.78
Bacteroides fragilis ATCC 25285	3.13	12.5	1.56
B. distasonis W-7	0.78	1.56	3.13
B. ovatus GAI 5630	25	50	25
B. thetaiotaomicron GAI 5536	25	50	25
B. vulgatus IFO 14291	0.39	0.39	1.56
Anaerorhabdus furcosus ATCC 25662	0.1	0.1	0.2
Tissierella praeacuta ATCC 25539	≦0.025	≦0.025	≦0.025
Porphyromonas asaccharolyticus ATCC 25260	3.13	12.5	3.13
Fusobacterium nucleatum ATCC 25586	0.05	0.1	0.39
Veillonella parvula GAI 5602	0.05	0.1	0.2

Agar dilution method (stamp method): Modified GAM agar (Nissui), Anaerobic system model 1024 (Forma), 10^4 cfu/spot, 37°C, 48 hours.

Table 3-1. Antibacterial activity of FK041 against clinical isolates.

Organism (No. of strains)	Antibiotic	MIC (μ g/ml)		
		Range	50%	90%
Staphylococcus aureus (40)	FK041	0.2 ~ 0.78	0.39	0.39
(MSSA)	CFDN	0.2 ~ 1.56	0.39	0.78
(methicillin: ≦6.25 μ g/ml)	CDTR	0.78 ~ 1.56	0.78	1.56
S. epidermidis (32)	FK041	0.1 ~ 50	0.2	1.56
(MSSE)	CFDN	0.1 ~ >100	0.2	6.25
(methicillin: ≦6.25 μ g/ml)	CDTR	0.2 ~ 50	1.56	3.13
Streptococcus pyogenes [a] (20)	FK041	≦0.025	≦0.025	≦0.025
	CFDN	≦0.025	≦0.025	≦0.025
	CDTR	≦0.025	≦0.025	≦0.025
S. agalactiae [a, c] (20)	FK041	≦0.025 ~ 0.05	≦0.025	≦0.025
	CFDN	≦0.025 ~ 0.05	0.05	0.05
	CDTR	≦0.025 ~ 0.05	0.05	0.05
S. pneumoniae [a, c] (33)	FK041	≦0.025 ~ 0.2	0.1	0.2
(PSSP)	CFDN	0.05 ~ 0.39	0.1	0.39
(benzylpenicillin: ≦0.1 μ g/ml)	CDTR	≦0.025 ~ 0.2	≦0.025	0.2
Enterococcus faecalis (20)	FK041	3.13 ~ 50	6.25	50
	CFDN	6.25 ~ >100	12.5	100
	CDTR	>100	>100	>100
Neisseria gonorrhoeae [b, c] (16)	FK041	≦0.025	≦0.025	≦0.025
	CFDN	≦0.025 ~ 0.05	≦0.025	0.05
	CDTR	≦0.025 ~ 0.2	0.05	0.2
Moraxella catarrhalis (21)	FK041	≦0.025 ~ 0.78	0.2	0.78
	CFDN	≦0.025 ~ 0.39	0.2	0.39
	CDTR	≦0.025 ~ 0.39	0.05	0.39
Escherichia coli (21)	FK041	≦0.025 ~ 1.56	0.05	0.2
	CFDN	0.1 ~ 1.56	0.2	0.39
	CDTR	0.1 ~ 6.25	0.39	0.78
Citrobacter freundii (21)	FK041	0.1 ~ >100	0.39	100
	CFDN	0.39 ~ >100	0.78	>100
	CDTR	0.39 ~ >100	1.56	100

Agar dilution method (stamp method): Mueller-Hinton agar, 10^4 cfu/spot, 37°C, 18 hours; [a] supplemented with 5% defibrinated horse blood ([b]chocolate agar), [c] 5% CO_2.

against *B. pertussis* and *H. influenzae*. Results of evaluation against 20 reference strains of anaerobic bacteria (Table 2) show that FK041 has potent and broad activity and that its activity is similar or superior to those of CFDN and CDTR against most anaerobic organisms tested except *Clostridium perfringens*.

Antibacterial Activity of FK041 against Clinical Isolates

The activities of FK041 against various clinical isolates were compared with those of CFDN and CDTR. Table 3 shows the drug concentration necessary to inhibit 50% (MIC_{50}) or 90% (MIC_{90}) of the test strains and the MIC range. FK041 exhibited potent activity against clinical

Table 3-2. Antibacterial activity of FK041 against clinical isolates.

Organism (No. of strains)	Antibiotic	MIC (μ g/ml) Range	50%	90%
Klebsiella pneumoniae (20)	FK041	≦0.025 ~ 100	0.05	0.39
	CFDN	0.1 ~ >100	0.1	1.56
	CDTR	0.1 ~ 100	0.2	1.56
Enterobacter cloacae (21)	FK041	0.2 ~ >100	0.39	>100
	CFDN	0.78 ~ >100	1.56	>100
	CDTR	0.39 ~ >100	0.78	>100
Serratia marcescens (20)	FK041	3.13 ~ >100	12.5	>100
	CFDN	3.13 ~ >100	50	>100
	CDTR	0.78 ~ 50	3.13	25
Proteus mirabilis (21)	FK041	≦0.025 ~ 0.05	≦0.025	0.05
	CFDN	0.05 ~ 0.2	0.1	0.1
	CDTR	0.05 ~ 0.39	0.1	0.2
P. vulgaris(21)	FK041	0.05 ~ 1.56	0.2	0.78
	CFDN	0.1 ~ 6.25	1.56	3.13
	CDTR	0.05 ~ 0.39	0.2	0.39
Morganella morganii (21)	FK041	≦0.025 ~ 12.5	0.2	6.25
	CFDN	0.78 ~ 25	6.25	25
	CDTR	0.1 ~ 3.13	0.1	3.13
P. rettgeri (20)	FK041	≦0.025 ~ 12.5	≦0.025	0.2
	CFDN	≦0.025 ~ 12.5	≦0.025	0.39
	CDTR	≦0.025 ~ 12.5	0.05	0.78
Pseudomonas aeruginosa (21)	FK041	12.5 ~ >100	>100	>100
	CFDN	50 ~ >100	>100	>100
	CDTR	12.5 ~ >100	>100	>100
Haemophilus influenzae [b,c] (59) (benzylpenicillin: ≦1.56 μ g/ml)	FK041	0.1 ~ 0.39	0.2	0.39
	CFDN	0.39 ~ 3.13	0.78	1.56
	CDTR	≦0.025 ~ 0.1	0.05	0.05
Bacteroides fragilis [d] (21)	FK041	3.13 ~ >100	3.13	50
	CFDN	12.5 ~ >100	12.5	100
	CDTR	1.56 ~ 100	3.13	25

Agar dilution method (stamp method): Mueller-Hinton agar, 10^4 cfu/spot, 37°C, 18 hours; [b] supplemented with 5% chocolate agar, [c] 5% CO_2; [d] GAM agar (Nissui), Anaerobic system model 1024 (Forma), 37°C, 24 hours.

isolates of Gram-positive bacteria, except *Enterococcus faecalis* , with MIC_{90}s equal to or less than 1.56 μg/ml. FK041 had advantages over CFDN and CDTR in activity against methicillin-sensitive *S. aureus* (MSSA), methicillin-sensitive *S. epidermidis* (MSSE), and *S. agalactiae*, and was similar to CFDN and CDTR against penicillin G-sensitive *S. pneumoniae* (PSSP) and *S. pyogenes*. The activity of FK041 was more potent than CFDN against clinical isolates of most Gram-negative organisms tested. Against *Neisseria gonorrhoeae*, *E. coli*, *K. pneumoniae*, *P. mirabilis*, *Proteus vulgaris*, and *Proteus rettgeri*, FK041 had good *in vitro* activity which was superior or similar to

Table 4. Antibacterial activity of FK041 against β-lactam-resistant and new quinolone-resistant clinical isolates.

Organism (No. of strains)	Antibiotic	MIC (μg/ml)			
		Range	50%	90%	Mean[c]
Staphylococcus aureus (21)	FK041	1.56 ~ >100	50	100	-
(MRSA)	CFDN	3.13 ~ >100	>100	>100	-
(methicillin: ≧12.5 μg/ml)	CDTR	6.25 ~ >100	100	>100	-
S. epidermidis (10)	FK041	1.56 ~ >100	50	100	-
(MRSE)	CFDN	0.2 ~ >100	>100	>100	-
(methicillin: ≧12.5 μg/ml)	CDTR	1.56 ~ >100	25	100	-
Streptococcus pneumoniae[a] (49)	FK041	0.05 ~ 3.13	3.13	3.13	-
(PRSP)	CFDN	0.1 ~ 12.5	6.25	6.25	-
(benzylpenicillin: ≧0.2 μg/ml)	CDTR	0.05 ~ 3.13	0.78	0.78	-
Haemophilus influenzae[b] (24)	FK041	0.2 ~ 0.78	0.2	0.39	-
(benzylpenicillin: ≧3.13 μg/ml)	CFDN	0.39 ~ 6.25	1.56	3.13	-
	CDTR	≦0.025 ~ 0.1	0.05	0.1	-
Escherichia coli (2)	FK041	≦0.025 ~ 0.78	-	-	≦0.14
(levofloxacin: ≧3.13 μg/ml)	CFDN	0.1 ~ 0.78	-	-	0.28
	CDTR	0.2 ~ 3.13	-	-	0.78
	LVFX	3.13 ~ 6.25	-	-	4.42
Klebsiella pneumoniae (4)	FK041	0.05 ~ 0.2	-	-	0.14
(levofloxacin: ≧3.13 μg/ml)	CFDN	0.2 ~ 0.39	-	-	0.33
	CDTR	0.39 ~ 0.78	-	-	0.55
	LVFX	3.13 ~ 25	-	-	7.43
Proteus mirabilis (2)	FK041	0.05 ~ 0.1	-	-	0.07
(levofloxacin: ≧3.13 μg/ml)	CFDN	0.1	-	-	0.10
	CDTR	0.2	-	-	0.20
	LVFX	>100	-	-	>100

Agar dilution method (stamp method): Mueller-Hinton agar, 10^4 cfu/spot, 37°C, 18 hours; [a] supplemented with 5% defibrinated horse blood ([b] chocolate agar), 5% CO_2. [c] Geometric mean.

that of CDTR. FK041 exhibited good MIC_{50}s, which were lower than those of CFDN and CDTR, but poor MIC_{90}s against *Citrobacter freundii* and *Enterobacter cloacae*. The compound, however, was inferior to CDTR in activity against *Moraxella catarrhalis*, *H. influenzae*, *Morganella morganii*, and *Serratia marcescens* and was inactive against *Pseudomonas aeruginosa*. Its activity against *Bacteroides fragilis* was slightly weaker than that of CDTR but stronger than that of CFDN.

Antibacterial Activity of FK041 against β-Lactam-resistant and Fluoroquinolone-resistant Organisms

Antibacterial activities of FK041 and reference compounds against β-lactam-resistant and fluoroquinolone-resistant organisms are shown in Table 4. FK041 was inactive against MRSA and MRSE like CFDN and CDTR. FK041 showed fairly good activity against PRSP with MIC range of 0.05~3.13 μg/ml and was superior to CFDN, but inferior to CDTR. Strains of *H. influenzae* resistant to benzylpenicillin were highly susceptible to FK041, which was more active than CFDN, but less active than CDTR. Against fluoroquinolone-resistant *E. coli*, *K. pneumoniae*, and *P. mirabilis*, FK041 had an excellent activity, superior to those of CFDN and CDTR, and there was no cross resistance with LVFX.

Table 5. Comparison of broth dilution MICs and MBCs of FK041.

Organism (No. of strains)	Antibiotic	Mean MBC (μg/ml)	Mean MIC (μg/ml)
Staphylococcus aureus (10)	FK041	0.20	0.20
	CFDN	0.48	0.36
	CDTR	0.73	0.68
Escherichia coli (10)	FK041	0.10	0.10
	CFDN	0.36	0.32
	CDTR	0.28	0.24
Haemophilus influenzae[a] (10)	FK041	0.11	0.10
	CFDN	1.03	0.42
	CDTR	0.02	0.01
Klebsiella pneumoniae (8)	FK041	0.11	0.09
	CFDN	0.20	0.16
	CDTR	0.33	0.25

MIC: Broth microdilution method, Mueller-Hinton broth, 10^6 cfu/ml, 37°C,18 hours; [a] supplemented with 5% Fildes enrichment. MBC: Killing 99.9% of the inoculum. Mean; Geometric mean.

Comparison of Broth Dilution MBCs and MICs of FK041

The microdilution broth MBCs and MICs of FK041 and the reference compounds against 8 to 10 strains each are shown in Table 5. The mean MBCs of FK041 were similar to the mean MICs against all organisms tested. The mean MBCs of FK041 against the organisms ranged from 0.10 to 0.20 μg/ml and were superior to those of CFDN and CDTR, except that it was inferior to CDTR in terms of MBC against *H. influenzae*.

Stability of FK041 to β-Lactamases Hydrolysis

Relative Vmax/*Km* values of FK041 for various β-lactamases were compared with those of CFDN and CDTR (Table 6). Based on these values, FK041 was highly stable to group 2a and 2b β-lactamase hydrolysis but was unstable to group 1b and 1c β-lactamase hydrolysis. The stability of FK041 to these enzymes was similar to those of CFDN and CDTR. FK041 exhibited good activity against group 2a and 2b β-lactamase-producing strains, but was inactive against group 1b β-lactamase-producing *E. coli*, reflecting its stability to these enzymes.

Affinity of FK041 for the PBPs of *S. aureus*, *E. coli* and *H. influenzae*

The affinities of FK041 and the reference compounds to PBPs of *S. aureus* 209P JC-1, *E. coli* NIHJ JC-2 and *H. influenzae* 7018 are expressed as concentrations of compounds required to reduce ^{14}C-benzylpenicillin binding by 50% (Table 7). For the main PBPs (3, 2 and 1) of *S. aureus*, the IC_{50}s of FK041 were 0.31 μg/ml or less, FK041 showed higher average binding affinities than CFDN and

Table 6. Stability of FK041 to β-lactamases.

β-Lactamase (Organism)	Relative stability[a] (MIC; μg/ml)		
	FK041	CFDN	CDTR
1b β-lactamase	34	107	45
(*Escherichia coli* FP950)	(>100)	(>100)	(50)
1c β-lactamase	141	137	25
(*Bacteroides fragilis* FP786)	(nd)	(nd)	(nd)
2b β-lactamase (TEM)	0.11	0.03	<0.05
(*Escherichia coli* FP1189)	(0.2)	(0.39)	(0.2)
2b β-lactamase	0.06	0.02	0.12
(*Klebsiella pneumoniae* FP1585)	(0.39)	(0.39)	(0.39)
2a β-lactamase	0.15[b]	0.05[b]	0.08[b]
(*Staphylococcus aureus* FP1191)	(0.1)	(0.05)	(0.39)

[a] Stability to β-lactamases was expressed as relative value (%) of Vmax/*Km* to that of cephaloridine for cephalosporinase or ampicillin for penicillinase. [b] *Ki* values substituted for *Km* values.

CDTR for these PBPs. Both FK041 and the reference compounds showed low affinity for PBP 4. Against the main PBPs of *E. coli*, FK041 inhibited PBP 3 to the greatest extent (IC_{50}<0.04 μg/ml), which was similar to that obtained with CDTR and CFDN. PBPs 1bs and 1a were inhibited at 0.19 and 0.39 μg/ml of FK041, respectively. These inhibitory activities of FK041 were similar to those of CDTR and superior to those of CFDN in the inhibition of PBP 1bs. FK041 inhibited PBPs 1A, 1B, 3A, and 3B of *H. influenzae* at 0.049 μg/ml or below. The affinities of FK041 for PBPs 3A and 3B were higher than those of CFDN, but lower than those of CDTR in accordance with their MICs against *H. influenzae*.

Discussion

Extensive investigations of new orally active cephem antibiotics such as CFIX and CFDN show that the introduction of a 2-carboxymethoxyimino aminothiazole side chain to the 7-position and a vinyl group to the 3-position of the cephem nucleus results in a significant enhancement of antibacterial activity against Gram-negative bacilli, stability to β-lactamases hydrolysis, and oral absorbability[14,15]. In addition, the introduction of hydroxyimino instead of 2-carboxymethoxyimino at the 7-position results in a great improvement in activity against Gram-positive organisms while maintaining good activity against Gram-negative organisms and oral absorbability[16]. FK041 is the best orally active chemotherapeutic agent to emerge from our research for an orally active cephem with a superior profile to CFDN. A pyrazole group at the 3-position has been shown to contribute to improvement in antibacterial activity. FK041 has a broad spectrums and has potent activity against common clinical isolates, *i.e.*, *S. aureus*, *S. pneumoniae*, *H. influenzae*, *E. coli*, *K. pneumoniae*, and *P. mirabilis*. The good antibacterial activity of FK041 can be explained by its high affinity for

Table 7. Affinity of FK041 for penicillin-binding proteins (PBPs).

Organism	PBPs	IC_{50} (μ g/ml)[a]		
		FK041	CFDN	CDTR
Staphylococcus aureus 209P JC-1	1	0.31	0.56	0.43
	2	0.10	0.10	0.20
	3	0.06	0.10	0.12
	4	7.7	1.8	7.2
	MIC (μ g/ml)	0.1	0.1	0.39
Escherichia coli NIHJ JC-2	1a	0.39	0.68	0.21
	1bs	0.19	2.6	0.14
	2	0.21	2.2	1.5
	3	<0.04	0.08	<0.04
	4	0.14	0.24	1.7
	5	>25	>25	>25
	6	16	20	>25
	MIC (μ g/ml)	0.05	0.2	0.1
Haemophilus influenzae 7018	1A	0.049	0.076	0.49
	1B	0.021	0.007	0.054
	2	0.39	>1	>1
	3A	0.016	0.074	<0.008
	3B	0.02	0.1	0.008
	4	0.5	0.54	>1
	5	>1	>1	>1
	6	0.47	0.08	0.93
	MIC (μ g/ml)	0.2	1.56	0.05

[a] Concentrations (μg/ml) of drug required to reduce ^{14}C-benzylpenicillin binding by 50%.

PBPs and its high stability to β-lactamases. The affinities of FK041 for the main PBPs of *S. aureus*, *E. coli*, and *H. influenzae* were higher than those of CFDN. This result suggests that the higher binding affinities of FK041 for the main PBPs are responsible for the more potent activities of FK041 compared to CFDN against most Gram-positive and Gram-negative organisms.

Penicillin G-resistant and multiple antibiotic-resistant pneumococci have been encountered with increasing frequency during the last decade[18,19]. Penicillin G-resistant isolates show increased resistance to other β-lactam antibiotics[18,19]. FK041 showed moderate activity against PRSP, with an MIC_{90} of 3.13 μg/ml, but the activity against PRSP was decreased by 16-fold in comparison with that against PSSP. Resistance to penicillin G is due to the development of altered forms of some of the PBPs, which thereby have decreased affinity for the antibiotic[20~23]. Increase in the MIC of FK041 against PRSP seems to be due to a decrease in affinity for PBPs 1A and 2X, which have shown to be associated with resistance to cephem antibiotics[20].

In comparison with CFDN, the most striking improvement of FK041 in terms of activity against clinically important pathogens is the elevated activity against *H. influenzae*. This improvement in activity against *H. influenzae* might be explained by the higher affinity of FK 041 for PBPs 3A and 3B. This observation also might explain the poorer activity of FK041 when compared with CDTR. Against murine pneumonia induced by intranasaly inoculated *H. influenzae* in an *in vivo* pharmacokinetic model simulating human plasma concentrations for oral administration of 100 mg, FK041, like CDTR, has been shown to be more effective than CFDN in reducing the number of viable bacteria in the lungs[9].

The results from preclinical evaluations performed thus far indicate that FK041 should be a potent orally active antibacterial agent.

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References

1) BILL, N. J. & J. A. WASHINGTON II.: Comparison of *in vitro* activity of cephalexin, cephradine, and cefaclor. Antimicrob. Agents Chemother. 11: 470~474, 1977
2) BUCK, R. E. & K. E. PRICE: Cefadroxil, a new broad-spectrum cephalosporin. Antimicrob. Agents Chemother. 11: 324~330, 1977
3) NEU, H. C. & K. P. FU: Cefatrizine activity compared with that of other cephalosporins. Antimicrob. Agents Chemother. 15: 209~212, 1979
4) KAMIMURA, T.; H. KOJO, Y. MATSUMOTO, Y. MINE, S. GOTO & S. KUWAHARA: *In vitro* and *in vivo* antibacterial properties of FK027, a new orally active cephem antibiotic. Antimicrob. Agents Chemother. 25: 98~104, 1984
5) SAIKAWA, I.; T. YASUDA, Y. WATANABE, Y. FUKUOKA, A. YOTSUJI, S. MINAMI, Y. YAMASHIRO, H. ARAKI & N. OOGAKE: *In vitro* and *in vivo* antibacterial properties of T-2588, a new oral cephem antibiotic. Chemotherapy (Tokyo) 34 (Suppl. 2): 66~84, 1986
6) MINE, Y.; T. KAMIMURA, Y. WATANABE, S. TAWARA, Y. MATSUMOTO, F. SHIBAYAMA, H. KIKUCHI, T. TAKAYA & S. KUWAHARA: *In vitro* antibacterial activity of FK482, a new orally active cephalosporin. J. Antibiotics 41: 1873~1887, 1988
7) MINE, Y.; Y. YOKOTA, Y. WAKAI, T. KAMIMURA, S. TAWARA, F. SHIBAYAMA, H. KIKUCHI & S. KUWAHARA: *In vivo* antibacterial activity of FK 482, a new orally active cephalosporin. J. Antibiotics 41: 1888~1895, 1988
8) SAKAMOTO, H.; T. HIROSE, S. NAKAMOTO, K. HATANO, F. SHIBAYAMA, H. KIKUCHI, Y. MINE & S. KUWAHARA: Pharmacokinetics of FK482, a new orally active cephalosporin, in animals. J. Antibiotics 41: 1896~1905, 1988
9) WATANABE, Y.; K. HATANO, Y. WAKAI, F. IKEDA, A. WAKA BAYASHI, K. OHTOMO, Y. MATSUMOTO, C. MORINAGA, S. MATSUMOTO, S. TAWARA, K. KAWABATA, H. YAMAMOTO, H. TAKASUGI, F. MATSUMOTO & S. KUWAHARA: FK041, a new orally active cephalosporin: *In vitro* and *in vivo* antibacterial activity. Program and Abstracts of the 36th Intersci. Conf. on Antimicrob. Agents Chemother., No. F131, p. 122, New Orleans, Sept. 15~18, 1996
10) MATSUMOTO, S.; H. SAKAMOTO, T. YAMAMOTO, F. MAEDA, S. TAWARA, H. KAGEYAMA, J. AZUMA, F. MATSUMOTO & S. KUWAHARA: FK041, a new orally active cephalosporin: Pharmacokinetics in animals and in healthy volunteers. Program and Abstracts of the 36th intersci. Conf. on Antimicrob. Agents Chemother., No. F132, p. 123, New Orleans, Sept. 15~18, 1996
11) National Committee for Clinical Laboratory Standards. Methods for dilution antimicrobial susceptibility test for bacteria that grow aerobically. NCCLS Document M7-A2. National Committee for Clinical Laboratory Standards, Villanova, PA, USA.
12) National Committee for Clinical Laboratory Standards. Methods for antimicrobial susceptibility testing of anaerobic bacteria. NCCLS Document M11-A2. National Committee for Clinical Laboratory Standards, Villanova, PA, USA.
13) SPRATT, B. G & A. B. PARDEE: Penicillin-binding proteins and cell shape in E. coli. Nature 254: 516~517, 1975
14) YAMANAKA, H.; K. KAWABATA, K. MIYAI, H. TAKASUGI, T. KAMIMURA, Y. MINE & T. TAKAYA: Studies on β-lactam antibiotics X. Synthesis and structure-activity relationships of 7β-[(*Z*)-2-(-2-amino-4-thiazolyl)-2-(carboxymethoxyimino)acetamido]cephalosporin derivatives. J. Antibiotics 39: 101~110, 1985
15) YAMANAKA, H.; H. TAKASUGI, T. MASUGI, H. KOCHI, K MIYAI & T. TAKAYA: Studies on β-lactam antibiotics VIII. Structure-activity relationships of 7β-[(*Z*)-2-carboxymethoxyimino-2-arylacetamido]-3-cephem-4-carboxylic acids. J. Antibiotics 38: 1068~1076, 1985
16) INAMOTO, Y.; T. CHIBA, K. SAKANE, T. KAMIMURA & T. TAKAYA: Studies on FK482. II. Synthesis and structure-activity relationships of 7β-[(*Z*)-2-(2-aminothiazol-4-yl)-2-substituted acetamido]-3-vinyl-3-cephem-4-carboxylic acid derivatives. Yakugaku Zasshi 110: 246~257, 1990
17) YAMAMOTO, H.; K. KAWABATA, S. TAWARA, H. TAKASUGI & H. TANAKA: FK041, a novel orally active cephalosporin: Synthesis and biological properties. J. Antibiotics 51: 683~687, 1998
18) APPELBAUM, P. C.: Antimicrobial resistance in *Streptococcus pneumoniae*: an overview. Clin. Infect. Dis. 15: 77~83, 1992
19) KLUGMAN, K. P.: Pneumococcal resistance to antibiotics. Clin. Microbiol. Rev. 3: 171~196, 1990
20) COFFEY, T. J.; M. DANIELS, L. K. MCDOUGAL, C. G. DOWSON, F. C. TENOVER & B. G. SPRATT: Genetic analysis of clinical isolates of *Streptococcus pneumoniae* with high-level resistance to expanded-spectrum cephalosporins. Antimicrob. Agents Chemother. 39: 1306~1313, 1995
21) MARKIEWICZ, Z. & A. TOMASZ: Variation in penicillin-binding protein patterns of penicillin-resistant clinical isolates of pneumococci. Antimicrob. Agents Chemother. 27: 405~410, 1989
22) abes, D.; S. NACHMAN & A. TOMASZ: Penicillin-binding protein families: Evidence for the clonal nature of penicillin resistance in clinical isolates of pneumococci. J. Infect. Dis. 159: 16~25, 1989
23) HAKENBECK, R.; M. TARPAY & A. TOMASZ: Multiple changes of penicillin-binding proteins in penicillin-resistant clinical isolates of *Streptococcus pneumoniae*. Antimicrob. Agents Chemother. 17: 364~371, 1980