CEFCLIDIN (E1040), A NOVEL CEPHALOSPORIN: LACK OF SELECTION OF β-LACTAMASE OVERPRODUCING MUTANTS IN AN *IN VITRO* PHARMACOKINETIC MODEL SYSTEM

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(Received for publication January 29, 1992)

The bactericidal activity of cefclidin (E1040), a new cephalosporin, against a clinical strain of *Citrobacter freundii* was compared with that of ceftazidime in a two-compartment *in vitro* pharmacokinetic model system designed to simulate plasma concentrations in humans for 12 hours after intravenous administration of a 1 g dose. Both cefclidin and ceftazidime showed rapid bactericidal activity against *C. freundii*. However, during the simulation of ceftazidime treatment, regrowth was observed after two hours and a subpopulation emerged which was resistant to ceftazidime. Neither regrowth nor the emergence of resistant mutants was observed with cefclidin during the 12-hour simulation. The ceftazidime-resistant mutants constitutively overproduced β-lactamase at levels which were about 500-fold higher than that of the parent wild-type strain. Against this β-lactamase overproducing mutant, no bactericidal activity of ceftazidime was observed in the *in vitro* model system, whereas the bactericidal activity of cefclidin was observed during the 12-hour period. The emergence of *Enterobacter cloacae* mutants derepressed for β-lactamase production was also observed with ceftazidime but not cefclidin. The affinity of cefclidin for the β-lactamase isolated from these mutants was lower than that of ceftazidime, and the kinetic parameters of enzymatic hydrolysis showed that cefclidin was hydrolyzed more slowly at a low concentration (0.2 μM) than was ceftazidime. It is suggested that the high activity of cefclidin against strains derepressed for β-lactamase plays a major role in the absence of emergence of resistant mutants.

β-Lactam antibiotics are extensively used in treatment of bacterial infections. However, Gram-negative bacteria commonly develop resistance during therapy with β-lactam antibiotics. Recently it has been observed that clinical isolates of some species, such as *Enterobacter cloacae*, *Citrobacter freundii*, and *Pseudomonas aeruginosa*, produce high levels of the chromosomal β-lactamase and are in turn resistant to the newer nonhydrolyzable β-lactams[1~4]. In these organisms the β-lactamase enzyme is normally inducible. Mutations which derepress the enzyme are known to lead to multiple β-lactam resistance[1]. The ability to select stably derepressed mutants depends on the specific β-lactam antibiotic. The β-lactam antibiotics most capable of exerting a selective pressure for resistance are the so-called third-generation cephalosporins and monobactams. These antibiotics are highly active against the wild-type cells, are generally poor inducers of the enzyme, and are poorly hydrolyzed by the β-lactamases[5]. A new cephalosporin, cefclidin (E1040), also possesses these characteristics and may be an active agent for the selection of the derepressed mutants[6,7]. It is expected, however, that β-lactamase-derepressed mutants do not occur during therapy with cefclidin since the compound is relatively active against the derepressed strains[6]. In order to confirm this assumption, the development of β-lactam resistance due to the derepression of chromosomal β-lactamase was investigated in an *in vitro* pharmacokinetic model system. The results are discussed with respect to the probable development of resistance *in vivo*.

Materials and Methods

Bacterial Strains

Citrobacter freundii E12075 WT and *Enterobacter cloacae* E10045 WT were recent clinical isolates, for which the MICs of β-lactam antibiotics were similar to the MIC_{50}s for the large number of isolates[6]. *C. freundii* E12075 SKR32 was a resistant mutant isolated from the strain E12075 WT after 12 hours in the *in vitro* pharmacokinetic model system with ceftazidime; *E. cloacae* E10045 SKR5 was from the E10045 WT strain.

The frequency of resistant mutants was expressed as the ratio of cfu (colony forming units) grown in the presence of the antibiotic to the cfu of the control, grown without an antibiotic. The plates were incubated at 37°C. About 20 to 30 colonies grown in the presence of each drug concentration were randomly picked and subcultured onto individual agar plates for single-colony isolation. The MIC of selected β-lactam antibiotics and the level of β-lactamase production were then determined for each isolate to identify resistant mutants.

Antibiotics and Media

Mueller-Hinton broth and agar (BBL Microbiology Systems, Cockeysville, Md., U.S.A.) were used for all the experiments. Cefclidin, cefpirome, and cefepime were synthesized at Eisai Co., Ltd., and other antibacterial agents used were obtained commercially: ceftazidime (Nippon Glaxo Co., Tokyo, Japan); cephalothin (Shionogi Pharmaceutical Co., Osaka, Japan); cefotaxime (Chugai Pharmaceutical Co., Tokyo, Japan); imipenem (Banyu Pharmaceutical Co., Tokyo, Japan).

Determination of Minimal Inhibitory Concentrations (MICs)

MICs were determined by an agar dilution method. Approximately 5×10^4 cfu per spot were inoculated onto Mueller-Hinton agar plates that contained two-fold serial dilutions of antibiotics. The MIC was considered to be the lowest concentration of antibiotic that completely inhibited visible growth after incubation for 18 hours at 37°C[6].

β-Lactamase Activity

β-Lactamase activity was measured by a spectrophotometric method[8]. The reaction mixture consisted of 3 ml of a substrate in 50 mM phosphate buffer (pH 7.0) and 50 μl of enzyme solution. Enzymes were partially purified from the sonic extracts by CM-Sephadex C-50 (Pharmacia, Stockholm, Sweden) ion-exchange chromatography. The enzyme reaction was carried out at 30°C. The spectral parameters used in this study were as follows: cefclidin, 264.5 nm, $\Delta\varepsilon = 8.64\ mM^{-1}\ cm^{-1}$; ceftazidime, 260 nm, $\Delta\varepsilon = 9.59\ mM^{-1}\ cm^{-1}$; cefpirome, 283 nm, $\Delta\varepsilon = 7.13\ mM^{-1}\ cm^{-1}$; cefepime, 264 nm, $\Delta\varepsilon = 8.24\ mM^{-1}\ cm^{-1}$; cefotaxime, 264 nm, $\Delta\varepsilon = 7.25\ mM^{-1}\ cm^{-1}$; cephalothin, 262 nm, $\Delta\varepsilon = 7.66\ mM^{-1}\ cm^{-1}$ [6]. The *Km* and *Ki* values were calculated from Lineweaver-Burk and Dixon plots, respectively. The protein concentration was estimated by the method of LOWRY *et al.*[9] with bovine serum albumin as the standard.

β-Lactamase Induction

An overnight culture was diluted 20-fold into 10 ml of fresh Mueller-Hinton broth and incubated with shaking at 37°C. After 2.5 hours of incubation, inducers were added. Incubation was continued for 2 hours, and the cells were harvested and washed twice with 50 mM phosphate buffer (pH 7.0)[10]. The cells were suspended in the same buffer and disrupted with a model 350 Sonifier (Branson Sonic Power Co., Danbury, Conn., U.S.A.) in an ice-water bath. Cell debris and unbroken cells were removed by centrifugation, and the resulting supernatant fluid was used as a crude enzyme. β-Lactamase activity was determined with 100 μM cephalothin as the substrate.

Isoelectric Focusing

Isoelectric focusing of the supernatants from centrifuged cell sonic extracts was performed on an Ampholine PAG plate (Pharmacia; pH range, 3.5~9.5) with a Pharmacia FBE-3000 unit at 10°C. Samples (10μg in 2 μl) were loaded and run at 30 Watt for 1.5 hours. β-Lactamase activity was visualized by

overlaying the gel with 1.5% agar containing 25 mM phosphate buffer (pH 7.0) and 50 μg of nitrocefin per ml. The pI value was determined by reference to the standard marker proteins.

Outer Membrane Proteins

Cell envelopes were prepared from cells grown in Mueller-Hinton broth to the late-exponential phase. Cells were harvested by centrifugation, suspended in 50 mM phosphate buffer (pH 7.0), and disrupted by sonication. The crude membrane fractions were obtained by ultracentrifugation of the disrupted cell suspension at $100{,}000 \times g$ for 60 minutes. Outer membranes were obtained by solubilization of the cytoplasmic membrane with 2% sodium lauroyl sarcosinate according to the method of SAWAI *et al.*[11], and were analyzed on 12% polyacrylamide gel containing 0.1% sodium dodecyl sulfate (SDS-PAGE)[12]. Protein bands were stained with Coomasie brilliant blue.

In Vitro Pharmacokinetic Model System

The bactericidal activity of each antibiotic was evaluated at 37°C in the two-compartment open model described by MURAKAWA *et al.*[13]. Concentrations and pharmacokinetic parameters of cefclidin in the model were based on the human plasma concentrations for 12 hours after a single iv (intravenous) injection of 1 g, as described by NAKASHIMA *et al.*[14]. Ceftazidime concentrations were also based on the human plasma concentrations for 12 hours after a single iv injection of 1 g[15]. The volume of medium in each compartment and flow rate were calculated from these parameters[13]. Contribution of protein-binding rates of cefclidin and ceftazidime to plasma levels was disregarded in this study since the binding rates of both compounds to plasma proteins were very low ($\leq 10\%$). Cefclidin and ceftazidime concentrations in the model were determined by microbiological assay with *Escherichia coli* E01174 (a clinical isolate) as the test strain.

Samples were taken at intervals for 12 hours, centrifuged, washed in saline solution, resuspended in the same volume of the diluent, and spread on Mueller-Hinton agar plates for estimation of the remaining cfu. Colonies grown on the agar plates were transferred by replica plating onto the cefclidin (0.10, 0.39 and 1.56 μg/ml) or ceftazidime (0.39, 3.13 and 25 μg/ml) containing agar plates and control plates to detect the resistant mutants. β-Lactamase overproducing mutants were detected by overlaying the control plates with 1% agar containing 0.016% phenol red (pH 9.0) and 0.3% cephalothin.

Results

Drug Concentration

The concentrations of cefclidin (Fig. 1) and ceftazidime (data not shown) measured during the experiments correlated well with the data from human volunteers[14,15]. No significant difference was found between the calculated human plasma concentration curve and the periodically determined concentrations in this model system.

Time-kill Curves in the *In Vitro* Kinetic Model System

Figure 2 shows the effect of cefclidin and ceftazidime on a *C. freundii* strain at antibiotic concentrations which simulate the concentration in human plasma after administration of a 1 g iv dose of antibiotic. The MICs of cefclidin and ceftazidime for strain E12075 WT were 0.10 and 0.39 μg/ml,

Fig. 1. Cefclidin concentrations in the *in vitro* kinetic model system designed to simulate human plasma levels after an iv dose of 1 g.

Solid line represents the calculated concentration-time curve of cefclidin for humans; points are the experimental data from samples periodically obtained from the *in vitro* model system.

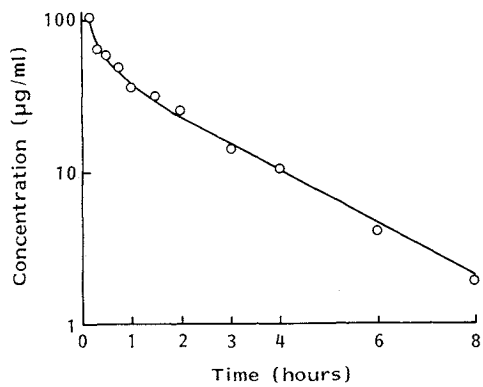
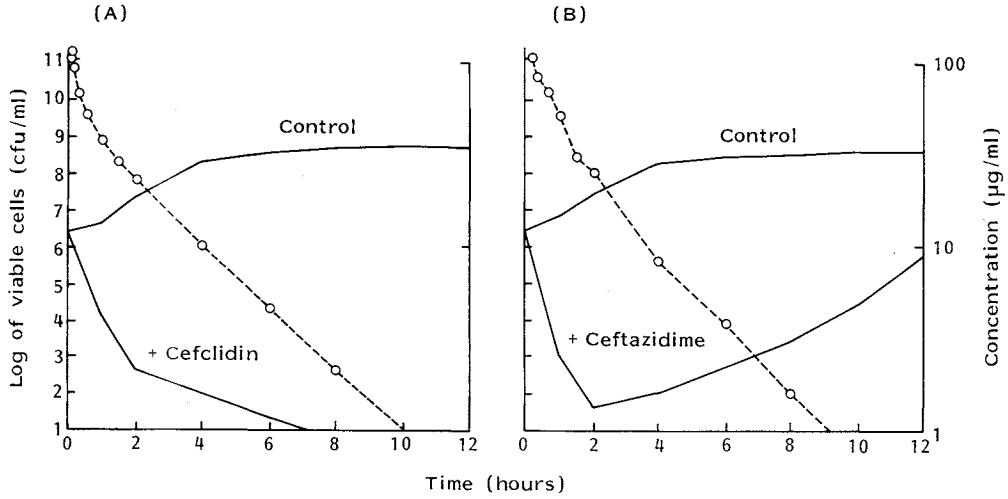


Fig. 2. Bactericidal activity of cefclidin (A) and ceftazidime (B) against *C. freundii* E12075 WT in the *in vitro* kinetic model system designed to simulate human plasma levels after a 1 g iv injection of each compound.

Solid lines represent the viable cell counts; broken lines represent the concentration of each compound.



respectively, at an inoculum size of 5×10^4 cfu. Both β-lactams showed very rapid bactericidal activity as demonstrated by a reduction of viable cell number of 4 to 5 orders of magnitude during the initial 2-hour period. After 6 hours with ceftazidime, the bacterial cell counts increased in spite of maintenance of concentrations above the MIC. The organism regrew to reach the initial bacterial concentration of about 10^6 cfu/ml at 12 hours. Based on analysis of subpopulations which emerged during the experiment, most of the ceftazidime survivors from 6 hours to 12 hours were mutants, resulting in elevated MIC values of ceftazidime and increased hydrolysis rate of cephalothin. One mutant strain from 12-hour subpopulations was isolated and designated SKR32. The MIC of ceftazidime for this mutant was 200 μg/ml, which was 512 times that for the parent wild-type strain (WT). In contrast, in the model of cefclidin, no viable cells were detected at intervals after 8 hours, and no regrowth was observed.

Against the ceftazidime-resistant mutant SKR32, ceftazidime showed no bactericidal activity in the *in vitro* model system (Fig. 3). In contrast, cefclidin still exhibited high bactericidal activity without apparent regrowth for 12 hours in the *in vitro* model system. Poor killing activity, however, was observed after 4 hours and the number of viable cells remained at about 10^3 per ml even at 12 hours. Susceptibility of the 12-hour subpopulations of SKR32 to antibiotics was unaffected.

Both cefclidin and ceftazidime showed rapid bactericidal activity against the wild-type strain of *Enterobacter cloacae* E10045, which was susceptible to cefclidin and ceftazidime with MICs of 0.10 and 0.39 μg/ml, respectively. The viable cell counts decreased to 10^{-4} to 10^{-5} of the initial inoculum by 2 hours (Fig. 4). However, regrowth was observed with ceftazidime and mutants resistant to ceftazidime developed after 4 hours. In the model of cefclidin, no viable cells were detected after 6 hours. One ceftazidime-resistant mutant, designated SKR5, from 12-hour subpopulations was chosen on the basis of drug susceptibility and β-lactamase production level. The MIC of ceftazidime for SKR5 was 100 μg/ml.

The MICs of β-lactam antibiotics for the wild-type strains and the ceftazidime-resistant mutants, SKR, are shown in Table 1. Both wild-type strains were susceptible to all β-lactams tested. The activities of ceftazidime and cefotaxime against the SKR mutants was 256- to 512-fold lower than those against

Fig. 3. Bactericidal activity of cefclidin (A) and ceftazidime (B) against *C. freundii* E12075 SKR32 in the *in vitro* kinetic model system designed to simulate human plasma levels after a 1 g iv injection of each drug.

Solid lines represent the viable cell counts; broken lines represent the concentration of each drug.

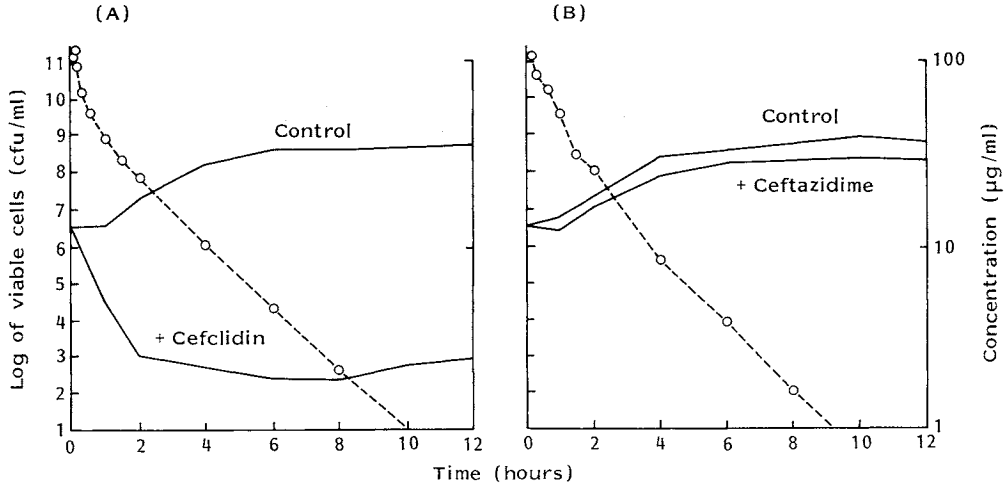


Fig. 4. Bactericidal activity of cefclidin (A) and ceftazidime (B) against *E. cloacae* E10045 WT in the *in vitro* kinetic model system designed to simulate human plasma levels after a 1 g iv injection of each drug.

Solid lines represent the viable cell counts; broken lines represent the concentrations of each drug.

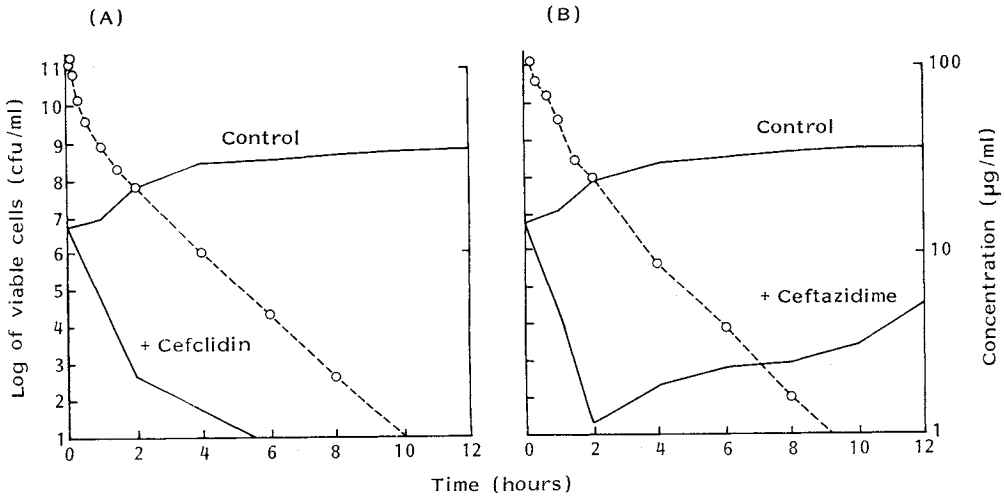


Table 1. Activity of β-lactam antibiotics against wild-type (WT) and β-lactam resistant mutants (SKR) of *C. freundii* and *E. cloacae* strains.

Organism and strain	Clone	MIC (μg/ml)					
		Cefclidin	Ceftazidime	Cefotaxime	Cefpirome	Cefepime	Imipenem
C. freundii E12075	WT	0.10	0.39	0.20	0.05	0.05	0.39
	SKR32	0.78	200	100	3.13	1.56	0.39
E. cloacae E10045	WT	0.10	0.39	0.20	0.05	0.05	0.39
	SKR5	0.39	100	50	0.78	0.20	0.39

the corresponding wild-type strains. Among the cephalosporins tested, MICs of cefclidin were least affected, although its activity was reduced four- to eight-fold against the SKR mutants. Imipenem showed no differences between the MICs for the wild-type strains and those for the corresponding SKR mutants.

β-Lactamase Production and Inducer Activity

In both wild-type strains E12075 WT and E10045 WT, β-lactamase production was hardly detectable. Addition of 0.39 and 1.56 μg/ml of imipenem resulted in marked induction (about 200- to 400-fold increases) of the chromosomal β-lactamase activity. The SKR mutants in the uninduced state exhibited levels of β-lactamase production about 500- to 1,000-fold higher than those of the wild-type strains. In contrast to wild-type strains, the levels of enzyme production in resistant mutants were not significantly enhanced by addition of imipenem (Table 2).

The ability of cefclidin and ceftazidime to induce enzymes in these strains was compared with that of imipenem (Table 2). Imipenem was the most potent inducer. Induction by cefclidin in these strains at ≤ 6.25 μg/ml was as low as that by ceftazidime. Although cefclidin was a poorer inducer than imipenem, induction by cefclidin at ≥ 25 μg/ml was higher than that by ceftazidime. Such dose-dependent increases in induction were also observed with ceftazidime.

Enzymes produced by each wild-type strain and the corresponding resistant mutant with and without imipenem were shown to be identical by isoelectric focusing. The enzymes produced by strains E12075 WT and E12075 SKR32 of *C. freundii* focused at pH 9.2 and the enzymes produced by the *E. cloacae* strains E10045 WT and E10045 SKR5 focused at pH 9.0. Satellite bands were observed in the SKR mutants and in the wild-type strains grown in the presence of imipenem. The apparent molecular weight of β-lactamase from each SKR mutant was determined to be about 39,000 (39 K protein) by SDS-PAGE.

Selection of Mutants

The ability of cefclidin and ceftazidime to select mutants stably derepressed for β-lactamase was

Table 2. Induction of β-lactamase in strains of *C. freundii* and *E. cloacae* by cefclidin and ceftazidime.

Organism	Inducer	β-Lactamase specific activity (nmol/minute/mg-protein) at the following inducer concentrations (μg/ml)[a]						
		0	0.10	0.39	1.56	6.25	25	100
C. freundii	None	13						
E12075 WT	Cefclidin		15	17	16	19	226	516
	Ceftazidime		21	17	18	17	15	26
	Imipenem		797	3,860	5,390			
C. freundii	None	6,770						
E12075	Cefclidin		5,840	6,130	6,510	6,280	5,960	10,400
SKR32	Ceftazidime		6,160	6,250	6,440	5,250	5,690	5,700
	Imipenem		7,040	9,700	12,500			
E. cloacae	None	47						
E10045 WT	Cefclidin		48	49	54	105	2,270	17,500
	Ceftazidime		43	54	55	88	469	1,530
	Imipenem		4,520	11,400	19,400			
E. cloacae	None	49,000						
E10045	Cefclidin		41,600	38,800	38,700	41,600	41,700	38,700
SKR5	Ceftazidime		45,700	47,100	45,200	42,300	44,500	39,000
	Imipenem		45,300	38,800	33,200			

[a] Determined with 100 μM cephalothin as the substrate.

assessed using the wild-type strains. Mutants derepressed for production of β-lactamase could be selected with frequencies of about 10^{-5} to 10^{-6} with both cefclidin and ceftazidime (Table 3). Although the mutation frequencies for ceftazidime and cefclidin were almost the same, the concentrations of antibiotics required to select mutants varied greatly. The highest concentration that allowed the occurrence of mutants from *C. freundii* E12075 WT was 0.20 μg/ml for cefclidin and 50 μg/ml for ceftazidime, and that from *E. cloacae* E10045 WT was 0.39 μg/ml for cefclidin and 50 μg/ml for ceftazidime. No mutants derepressed

Table 3. Selection of resistant mutants on Muellar-Hinton agar plates[a].

Strain	Compound for selection	Mutation frequency with compound at the following concentrations (μg/ml):					
		100	50	25	12.5	6.25	3.13
C. freundii	Cefclidin	—[b]	—	—	—	—	—
E12075 WT	Ceftazidime	—	2×10^{-7}	2×10^{-6}	4×10^{-6}	4×10^{-6}	4×10^{-6}
E. cloacae	Cefclidin	—	—	—	—	—	—
E10045 WT	Ceftazidime	—	1×10^{-7}	2×10^{-6}	2×10^{-6}	1×10^{-5}	1×10^{-5}

Strain	Compound for selection	Mutation frequency with compound at the following concentrations (μg/ml):				
		1.56	0.78	0.39	0.20	0.10
C. freundii	Cefclidin	—	—	—	2×10^{-6}	2×10^{-6}
E12075 WT	Ceftazidime	4×10^{-6}	4×10^{-6}	4×10^{-6}	++[c]	++
E. cloacae	Cefclidin	—	—	1×10^{-7}	2×10^{-6}	4×10^{-6}
E10045 WT	Ceftazidime	1×10^{-5}	1×10^{-5}	1×10^{-5}	5×10^{-4}	++

[a] The frequency of resistant mutants is expressed as the ratio of cfu grown in the presence of the antibiotic to the cfu of the control (grown without an antibiotic).
[b] —, Not detected ($<10^{-9}$).
[c] ++, Colonies of resistant mutants were unable to be selected ($>10^{-3}$).

Table 4. Kinetic parameters of cephalosporins for β-lactamases from *C. freundii* E12075 SKR32 and *E. cloacae* E10045 SKR5.

Enzyme produced by:	Cephalosporin	Relative V_{max}[a]	*Km* (μM)	*Ki* (μM)	Relative rate of hydrolysis at 0.2 μM[b]
C. freundii	Cephalothin	100	16.5		100
E12075 SKR32	Cefclidin	0.0106	385	>400	0.00046
	Ceftazidime	0.0016	0.91	0.73	0.0241
	Cefotaxime	0.0015	—[c]	0.0034	0.123[d]
	Cefpirome	0.0416	40.0	35.6	0.0173
	Cefepime	0.0060	159	126	0.00063
E. cloacae	Cephalothin	100	34.4		100
E10045 SKR5	Cefclidin	0.0128	313	>400	0.00141
	Ceftazidime	0.0068	10.5	8.20	0.0220
	Cefotaxime	0.0053	—	0.034	0.784
	Cefpirome	0.0183	145	128	0.00436
	Cefepime	0.0063	122	262	0.00178

[a] Relative value, calculated by normalizing to the V_{max} value with cephalothin.
[b] Calculated relative rate of hydrolysis at a substrate concentration of 0.2 μM.
[c] —, *Km* values could not be obtained for cefotaxime, because the values were much smaller than the substrate concentrations used.
[d] Calculated with *Ki* instead of *Km*.

Table 5. Effect of β-lactamase induction on cephalosporin MICs.

β-Lactam	MIC (μg/ml) for:			
	C. freundii E12075 WT		*E. cloacae* E10045 WT	
	Uninduced	Induced[a]	Uninduced	Induced
Cefclidin	0.10	0.10	0.10	0.10
Ceftazidime	0.39	3.13	0.39	3.13
Cefotaxime	0.20	1.56	0.39	3.13
Cefpirome	0.05	0.05	0.05	0.10
Cefepime	0.05	0.05	0.05	0.10

[a] After 2 hours of growth at 1.56 μg/ml imipenem.

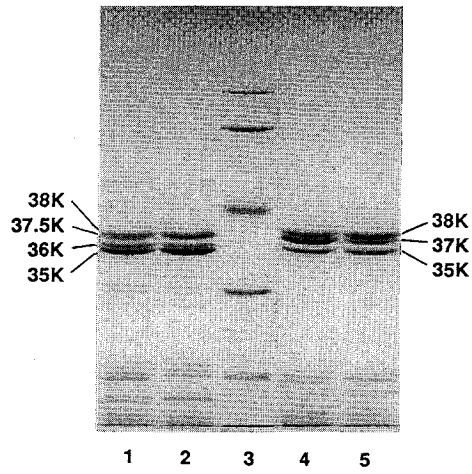
for β-lactamase were isolated from these strains using imipenem (data not shown).

Enzyme Kinetics

The V_{max} values of cefclidin were about 2 to 6 times greater than those of ceftazidime and cefotaxime for *C. freundii* and *E. cloacae* enzymes. The V_{max}s of cefclidin were about 10^{-4} times those of cephalothin (Table 4). Furthermore, the *Km* values of cefclidin was more than 30 times higher than those of ceftazidime. For cefotaxime, the exact *Km* values could not be obtained because those values were much smaller than the substrate concentrations used. The *Ki* values of cefclidin were more than 50 times higher than those of ceftazidime, approximately 4 orders of magnitude higher than those of cefotaxime, and almost similar to those of cefepime. The high *Km*s and *Ki*s of cefclidin indicate that this compound has the lower affinity for the enzymes than cefotaxime or ceftazidime. The rates of hydrolysis of the cephalosporins at 0.2 μM (approximately 0.10 μg/ml)[16], a concentration of compound nearly equal to that required to inhibit susceptible strains of *C. freundii* and *E. cloacae*, were calculated from V_{max}s and *Km*s (or *Ki*s for cefotaxime) using the Michaelis-Menten equation. At 0.2 μM, cefclidin was hydrolyzed at least two orders of magnitude more slowly than cefotaxime and about 10 to 50 times more slowly than ceftazidime by *C. freundii* and *E. cloacae* enzymes. The rates of hydrolysis of cefepime and cefpirome were about equal to and faster than that of cefclidin, respectively.

Fig. 5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of outer membrane proteins from *E. cloacae* E10045 WT (lane 1), *E. cloacae* E10045 SKR5 (lane 2), *C. freundii* E12075 WT (lane 4), and *C. freundii* E12075 SKR32 (lane 5).

Molecular weight standards are in lane 3; phospholipase b (94 K), bovine serum albumin (67 K), ovalbumin (43 K), carbonic anhydrase (30 K), and soybean trypsin inhibitor (20.1 K). 38 K, 38,000 molecular weight.



Effect of β-Lactamase Induction on Cephalosporin MICs

MICs of cephalosporins for *C. freundii* E12075 WT and *E. cloacae* E10045 WT containing inducible β-lactamase were determined pre- and post-induction (Table 5). In both strains, the induced level of β-lactamase was about 400 times higher than in the uninduced state (Table 2). The higher β-lactamase

levels in both strains were accompanied by 8- to 16-fold increases in the ceftazidime and cefotaxime MICs. Cefclidin MICs were unaffected by induction of *C. freundii* E12075 WT and *E. cloacae* E10045 WT enzymes.

Membranes

In the outer membrane fractions of the SKR mutants of *C. freundii* E12075 and *E. cloacae* E10045, slight differences between the relative amounts of 38 K and 37 K proteins of E12075, and 38 K and 37.5 K proteins of E10045 were observed, compared with those of the corresponding wild-type strains. No other marked changes in the outer membrane proteins were observed (Fig. 5).

Discussion

All Gram-negative organisms possess chromosomally mediated β-lactamases[5,10]. In some organisms, such as *C. freundii* and *E. cloacae*, the enzyme is inducible. Overproduction (derepression or induction) of the enzyme in these organisms causes resistance to multiple β-lactam antibiotics. In this study, it was observed that increased β-lactamase levels, due to either induction or derepression, elevated the MICs of ceftazidime and cefotaxime. Clinical isolates resistant to multiple β-lactams are predominantly derepressed mutants[1~4]. The so-called third-generation cephalosporins, such as ceftazidime, appear to be poor inducers of β-lactamase but very effective agents for selection of derepressed mutants[5]. Cefclidin, a newer cephalosporin, is also a poor inducer. Derepressed mutants were selected with the same frequency (about 10^{-6}) on agar plates by cefclidin as by ceftazidime, although the concentrations of cefclidin which permitted selection of mutants were lower than those of ceftazidime. For example, no mutants were detected when cefclidin was used at concentrations greater than 0.78 μg/ml, whereas mutants were easily detected with ceftazidime even at a concentration of 50 μg/ml. This difference was also observed in the time-killing curves of cefclidin and ceftazidime against *C. freundii*[6], where mutants derepressed for β-lactamase production were observed at concentrations higher than MICs with both compounds. The concentration of cefclidin that gave the maximal killing rate with no emergence of mutants was only two-fold higher than the MIC; the concentration of ceftazidime required for the maximal killing rate with no emergence of mutants was 16-fold higher than the MIC. These data suggest that cefclidin may allow selection of resistant mutants derepressed for β-lactamase during therapy. However, in this study, there was no evidence that cefclidin caused emergence of resistant mutants in the *in vitro* model simulating human plasma concentrations. In contrast, mutants were detected with ceftazidime in the *in vitro* model. Both compounds showed almost the same pharmacokinetic profile. The reason that cefclidin did not select for resistant mutants in the *in vitro* model, although they were selected on agar plates containing cefclidin, may be as follows. Since cefclidin showed activity against resistant strains derepressed for β-lactamase production and the concentrations of cefclidin were maintained above the MICs (0.39 to 0.78 μg/ml) for the resistant mutants at least until 8 hours in the kinetic model, this type of mutants would be unable to overcome cefclidin activity and therefore no emergence of resistant mutants would be observed. It has been reported that resistant mutants derepressed for β-lactamase production are not only obtained under laboratory conditions, but also can arise during β-lactam therapy[1~4,17]. In this regard, cefclidin has potential benefit in the therapy of Gram-negative bacterial infections.

The high activity of cefclidin against strains derepressed for β-lactamase production was suggested to result mainly from the low affinity of this compound for β-lactamase, coupled with the high resistance to enzymatic hydrolysis, which is consistent with previous observations[7,18]. The results of the induction experiments (Table 5) also confirm the greater impact of increased β-lactamase levels on the MICs of ceftazidime and cefotaxime than on the MIC of cefclidin, since the association of elevated β-lactamase levels with the magnitude of the MIC increase was common to both induced cells and derepressed mutants.

Cefclidin binds preferentially to penicillin-binding protein (PBP) 3, similar to other newer cephalosporins. The affinity of cefclidin for PBP 3 of *Escherichia coli* is lower than those of ceftazidime, cefepime, and cefpirome[6,19]. It is therefore assumed that the behavior of cefclidin to PBPs is not involved in this mechanism of resistance. In the case of compounds with an extremely low affinity for β-lactamase

like cefclidin, binding of the compounds to PBPs is likely to be much less affected by the periplasmatically located β-lactamase than in compounds with high affinity for β-lactamase. Indeed, the resistance of cefclidin to β-lactamase at low drug concentrations was much higher than those of ceftazidime and cefotaxime, and almost equal to that of cefepime.

Resistant mutants selected by the newer cephalosporins were observed to have not only alteration in β-lactamase expression, but also alteration in expression of outer membrane proteins or in penetration of β-lactams through the outer membrane[20~26]. Altered expression of outer membrane proteins enhanced the resistant level to β-lactams. However, in ceftazidime-resistant mutants obtained in this study, derepression for β-lactamase production was observed but there was no marked reduction in the outer membrane proteins. Alterations in β-lactamase production alone may be sufficient for bacteria to overcome β-lactams in the *in vitro* pharmacokinetic model simulating plasma concentrations. At extremely higher concentrations, such as those in the urinary tract which is the main route for the elimination of β-lactams, the overproduction of β-lactamase may be insufficient, and the alteration in outer membrane proteins may be required. Compounds having a dipolar ion, such as cefclidin, can permeate the outer membrane of Gram-negative bacteria more rapidly than other newer cephalosporins[18]. The MICs of cefclidin were not affected for the *ompR E. coli* strains with enzyme production[7]. High resistance of cefclidin to β-lactamase at low drug concentrations was displayed in the bacterial environment of lowered permeability.

Cefepime and cefpirome, new cephalosporins, also possess the low affinity for chromosomal β-lactamase and showed high activity against strains producing high levels of β-lactamase[27,28], and were reported to select no resistant mutants derepressed for β-lactamase[29,30]. However, it has recently been reported that cefpirome and ceftazidime are equally capable of selecting mutants of Gram-negative bacteria *in vitro* and *in vivo* with decreased susceptibility to β-lactams[20]. Since the *in vitro* pharmacokinetic model experiments clearly showed that ceftazidime (in this study) and cefotaxime[31] were ineffective against strains of *Citrobacter* and *Enterobacter* species, the results of sensitivity testing of these species and mutation frequencies may be misleading. In order to predict the development of resistance after β-lactam therapy, MICHÉA-HAMZEHPOUR *et al.*[32] have recently proposed the determination of resistance emerging in broth containing at least 16 times the MIC of the antibiotic tested. However, it is more useful to investigate the emergence of resistant mutants in the *in vitro* pharmacokinetic model for prediction of clinical therapeutic significance, since it is also able to evaluate the time-killing action of the antibiotic, although it is very time-consuming and expensive[33].

In conclusion, low β-lactamase hydrolysis at low drug concentrations and the apparent low affinity for the β-lactamase of cefclidin help to explain its potent antibacterial activity against cephalosporinase-overproducing Gram-negative bacteria, and it is suggested that the development of resistance during clinical use of cefclidin is unlikely when adequate plasma levels are maintained.

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