MECHANISM OF ACTION OF THE NEW ORALLY ACTIVE CEPHALOSPORIN FK027

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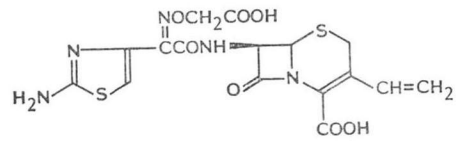
(Received for publication January 5, 1984)

The mechanism of action of a new orally active cephalosporin, FK027, was compared to that of cephalexin and cefaclor to elucidate its excellent antibacterial activity against Gram-negative bacteria. FK027 showed very high affinity for the penicillin-binding proteins (PBPs) 3, 1a and 1bs of *Escherichia coli* whereas cephalexin showed fairly high affinity for PBPs 1a, 4 and 3. The ability of FK027 to penetrate the outer membranes of *E. coli* and *Enterobacter cloacae* was less than that of cephalexin and cefaclor. However, FK027 was extremely stable to both plasmid-mediated penicillinases and chromosomal β-lactamases except the *Bacteroides fragilis* enzyme and its stability was superior to that of cephalexin and cefaclor. These results indicate that the potent antibacterial activity of FK027 is based on its enhanced affinity for the target enzymes and its high stability to β-lactamases.

FK027 (Fig. 1) is the first of the what is called third generation orally active cephalosporins. Although FK027 is less active against staphylococci than existing orally active β-lactam antibiotics, its activity is similar to cefaclor against streptococci and is far more potent than other orally active β-lactams against a wide range of Gram-negative bacteria. The purpose of this report is to elucidate the molecular basis of the potent antibacterial activity of FK027 against Gram-negative bacteria. Using mainly *Escherichia coli* systems, we investigated the relationship between its antibacterial activity and its affinity for the target enzymes (penicillin-binding proteins), its ability to penetrate the outer membrane and its stability to β-lactamases.

Fig. 1. Chemical structure of FK027.
(6*R*,7*R*)-7-[(*Z*)-2-(2-Amino-4-thiazolyl)-2-(carboxymethoxyimino)acetamido]-8-oxo-3-vinyl-5-thia-1-azabicyclo[4,2,0]oct-2-ene-2-carboxylic acid



Materials and Methods

Antibiotics

The antibiotics used were FK027, ceftizoxime, benzylpenicillin (Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan), cephalexin, cefaclor (Eli Lilly & Co., Indianapolis, Ind.) ampicillin and amoxicillin (Beecham Pharmaceuticals, Betchworth, England). [^{14}C]Benzylpenicillin (51 mCi/mmol) was purchased from Amersham International Ltd., Buckinghamshire, England.

Bacterial Strains

Escherichia coli MC1061 was obtained from Dr. K. FONG of the National Institute of Environmental Health Sciences, U.S.A. *E. coli* CSH2 (Nal^r), 13 strains of *E. coli* CSH2 harboring R-plasmids specifying ampicillin resistance and 14 chromosomally ampicillin-resistant strains of *E. coli* were provided by Dr. T. YOKOTA of Juntendo University. *E. coli* NIHJ and *Staphylococcus aureus* 209P were laboratory reference strains. All other strains used were isolated from clinical specimens and stocked in our labora-

tory. The clinical isolates of *E. coli* possessing ampicillin-resistance plasmids were identified by the conventional conjugation test[1]. Eleven β-lactam antibiotic-resistant strains selected from the clinical isolates were used to prepare the β-lactamases. *Enterobacter cloacae* No. 91 and *E. coli* MC1061 (pCF3) were used as test strains to evaluate the outer membrane permeability. Plasmid pCF3 is a pMB9 derivative that contains a chromosomal cephalosporinase gene of *E. coli* No. 253, a cephem-resistant clinical isolate (I. ARAMORI, H. KOJO, M. NISHIDA, S. KUWAHARA and S. GOTO, manuscript in preparation).

Antibiotic Susceptibility Testing

The minimum inhibitory concentrations (MICs) of the test antibiotics were determined by the agar dilution method. One hundred-fold dilutions of overnight cultures in Mueller-Hinton broth containing 10^4 colony forming units (unless specified otherwise) were inoculated with a multipoint replicating apparatus onto Mueller-Hinton agar plates containing serial two-fold dilutions of an antibiotic. After incubation at 37°C for 18~20 hours, the lowest concentration that inhibited macroscopic colonial growth was regarded as the MIC.

Assay of Affinity for Penicillin-binding Proteins

The affinity of FK027 and cephalexin for the PBPs of *E. coli* NIHJ and *S. aureus* 209P was assayed by a modification of SPRATT's method[2,3] as described previously[4].

Preparation of β-Lactamases

The cells were grown at 37°C in Trypticase-soy broth (GAM-broth for *Bacteroides fragilis*) to which ampicillin was added as an inducer when necessary. Exponentially growing cells were harvested by centrifugation, washed once, and suspended in 0.067 M potassium phosphate buffer (pH 7.0). The cell suspensions were sonicated at 20 kilocycles for 10 minutes. After cellular debris were removed by centrifugation, the supernatant was subjected to gel filtration on a Sephadex G-100 column. The column was equilibrated with 0.067 M phosphate buffer (pH 7.0) and eluted with the same buffer. The enzyme fractions were pooled and stored at −20°C.

Assay of β-Lactamase Activity

β-Lactamase activity was determined with a Hitachi 200A spectrophotometer equipped with a thermostatted cell holder. The enzyme was mixed in a 1-cm quartz cuvette with 150 μg of substrate and 200 μmol of phosphate buffer (pH 7.0) to make a final volume of 3 ml and was incubated at 37°C. The rate of hydrolysis of the β-lactam ring was followed by the change in absorption at 240 nm for ampicillin and at 260 nm for the cephalosporins. The relative initial rate of hydrolysis was expressed as a percent of hydrolysis of cephaloridine for cephalosporinase and of ampicillin for penicillinase.

Determination of Permeability Coefficients

The permeability coefficients of cephaloridine, cephalexin and cefaclor were determined by the method of ZIMMERMANN and ROSSELET[5] while those of FK027 and ceftizoxime were determined as reported previously[6]. The former method determines the permeability coefficients of β-lactam antibiotics by utilizing their susceptibility to the β-lactamase in the periplasm, and the latter by utilizing their inhibitory activity against β-lactamase.

Results

Affinity of FK027 for the Penicillin-binding Proteins of *E. coli* and *S. aureus*

FK027 is an orally active cephalosporin with a potent antibacterial activity against a wide range of Gram-negative bacteria[7]. To elucidate the mechanism of the antibacterial activity of FK027, its affinity for the target enzymes, namely penicillin-binding proteins (PBPs), was first determined by competition with [^{14}C]benzylpenicillin and compared with that of cephalexin, a representative oral cephalosporin. FK027 showed very high affinity for PBPs 3 (ID_{50}, 0.2 μg/ml), 1a (0.2 μg/ml) and 1bs (0.2 μg/ml), moderate affinity for PBPs 6 (13 μg/ml) and 2 (16 μg/ml), and low affinity for PBPs 4 (125 μg/ml)

Fig. 2. Competition of FK027 and cephalexin for the penicillin-binding proteins of *E. coli* NIHJ.
Concentrations of cephalosporins added to the membrane fractions were FK027 (a)~(e): 125, 25, 5, 1, 0.2 μg/ml respectively, (f) control without antibiotic, cephalexin (g)~(k): 0.2, 1, 5, 25, 125 μg/ml respectively.

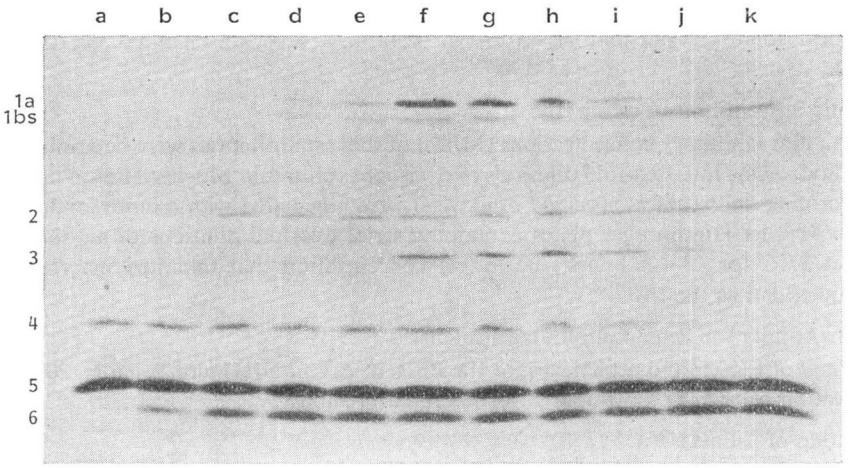
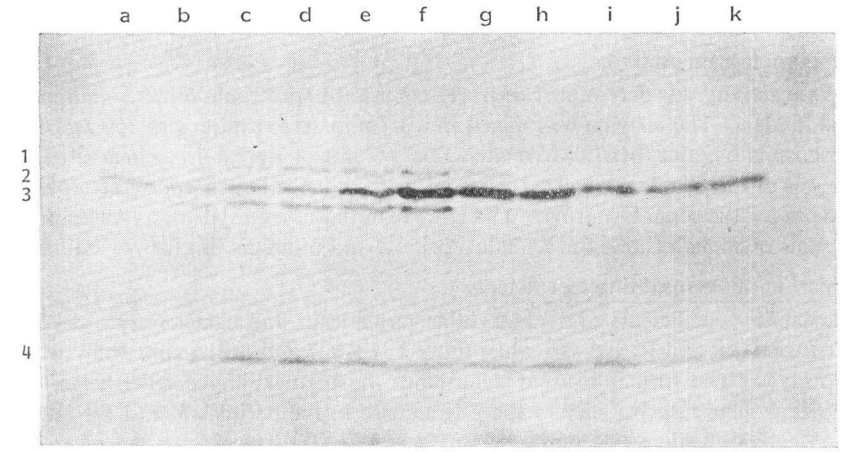


Fig. 3. Competition of FK027 and cephalexin for the penicillin-binding proteins of *S. aureus* 209P.
Concentrations of cephalosporins added to the membrane fractions were FK027 (a)~(e): 125, 25, 5, 1, 0.2 μg/ml respectively, (f) control without antibiotic, cephalexin (g)~(k): 0.2, 1, 5, 25, 125 μg/ml respectively.



and 5 (125 μg/ml), while cephalexin showed fair affinity for PBPs 1a (0.83 μg/ml), 4 (3.0 μg/ml) and 3 (8.7 μg/ml), but quite low affinity for the other PBPs including PBP 1bs (Fig. 2). PBP 1bs and its compensating enzyme PBP 1a are thought to function in cell elongation, whereas PBP 3 is thought to function in septum formation[8,9]. The affinity of β-lactam antibiotics for either type of the PBPs were well correlated with their MICs, suggesting that these PBPs are essential targets for β-lactam antibiotics[10,11]. Consequently, the excellent antibacterial activity of FK027 for *E. coli* can be explained by its strong affinity for PBPs 3, 1a and 1bs whereas the MIC value of cephalexin seems to be based on its affinity for PBP 3 alone. The affinity of FK027 for *S. aureus* PBPs was also evaluated (Fig. 3) since the antibacterial activity of FK027 against *S. aureus* is less potent in contrast with that against the Gram-negative bacteria.

The affinity of FK027 for *S. aureus* PBP 2 (ID_{50}, 0.2 μg/ml) was higher than that of cephalexin (1.7 μg/ml). But the affinity of FK027 for *S. aureus* PBPs 1 (2.9 μg/ml) and 3 (6.2 μg/ml) was comparatively lower than that of cephalexin (IDs_{50} for 1 and 3, 0.2 μg/ml). The MICs of β-lactam antibiotics for *S. aureus* were reported to correlate with their affinity for either PBP 1 or 3[12]. Accordingly, the lower antibacterial activity of FK027 against *S. aureus* is likely to result from its lower affinity for PBPs 1 and 3.

Ability of FK027 to Penetrate the Outer Membrane of *E. coli* and *E. cloacae*

The ability of FK027 to penetrate the outer membrane of *E. coli* and *E. cloacae* was evaluated by the method which we devised and reported previously[6,13]. This method is characterized by the capability to estimate the outer membrane permeability to β-lactamase-stable β-lactam antibiotics. In this method, the permeability coefficients of β-lactamase-stable β-lactam antibiotics are determined by measuring their ability to inhibit hydrolysis of a substrate by periplasmic β-lactamase. This measurement requires a high β-lactamase producer as a test strain. This prerequisite was satisfied by the clinical isolate of *E. cloacae*, but not by the strains of *E. coli* including those possessing ampicillin-resistance plasmids. Accordingly, we prepared an *E. coli* strain possessing a plasmid coding for the cephalosporinase gene by gene manipulation and used it as a test strain (I. ARAMORI, H. KOJO, M. NISHIDA, S. KUWAHARA and S. GOTO, manuscript in preparation). The outer membrane permeability to cephalexin and cefaclor was determined by the method of ZIMMERMANN and ROSSELET[5]. The ability of FK027 to penetrate the outer membranes of both *E. coli* and *E. cloacae* was one order of magnitude weaker than that of cephalexin and cefaclor (Table 1). *E. coli* and *E. cloacae* showed similar profiles of outer membrane permeability to the test cephalosporins though some differences could be seen between the two strains.

Table 1. Outer membrane permeability of *E. coli* and *E. cloacae* to FK027 and 4 other cephalosporins.

Antibiotic	Organism	
	E. coli	*E. cloacae*
FK027[b]	1.2×10^{-2} [a]	1.3×10^{-1}
Cefaclor[c]	4.5×10^{-1}	1.1×10^{0}
Cephalexin[c]	2.1×10^{-1}	5.7×10^{-1}
Ceftizoxime[b]	7.1×10^{-2}	1.3×10^{-1}
Cephaloridine[c]	1	1

[a] Permeability coefficients were expressed as a ratio to that of cephaloridine.
[b] Determined by the method of KOJO *et al.*[6].
[c] Determined by the method of ZIMMERMANN and ROSSELET[5].

Stability of FK027 to β-Lactamases

The stability of FK027 to β-lactamases was compared with that of five β-lactam antibiotics; cephalexin, cefaclor, ceftizoxime, cephaloridine and ampicillin. According to the classification proposed by RICHMOND and SYKES[14], nine distinct types of β-lactamases were selected from clinical isolates and used for assay, together with the enzymes from *B. fragilis* and ampicillin-resistant *S. aureus*. The relative initial rates of hydrolysis of the β-lactam antibiotics were expressed as percent of hydrolysis of cephaloridine for cephalosporinase and of ampicillin for penicillinase. FK027 was extremely stable to all types of β-lactamases except *B. fragilis* (Table 2). On the contrary, cephalexin as well as cefaclor were markedly unstable to the cephalosporinases. We examined how this excellent stability of FK027 to the β-lactamases was reflected in its antibacterial activity, using clinical isolates of *E. coli*. Ampicillin-resistant strains of *E. coli* can be devided into two groups; 1) strains possessing ampicillin-resistance plasmids and 2) chromosomally resistant strains. The antibacterial activity of FK027 against both types of resistant strains was compared with that of cephalexin, cefaclor and amoxicillin. As expected from the high stabi-

Table 2. Stability of FK027 and 5 other β-lactam antibiotics to β-lactamases.

Antibiotic[a]	Relative rate of hydrolysis by each class of β-lactamase[b]										
	Ia(1)	Ia(2)	Ib	Ic	Id	II	III	IV	V	*B. fragilis*	*S. aureus*
FK027	4.4	0.3	1.0	2.6	0.9	<0.1	0.4	<0.1	0.1	12	<0.1
Cefaclor	140	45	102	290	31	<0.1	2.7	3.2	0.3	46	2.2
Cephalexin	41	45	46	37	35	<0.1	0.6	0.3	0.2	6.9	0.3
Ceftizoxime	4.6	0.4	0.8	2.8	1.3	<0.1	0.8	<0.1	7.7	2.0	0.1
Cephaloridine	100	100	100	100	100	1.4	28	23	4.5	100	0.2
Ampicillin	8.5	0.5	4.0	36	<0.2	100	100	100	100	1.5	100

[a] Substrate concentration: 50 μg/ml.

[b] Relative initial velocity: Cephaloridine = 100 for cephalosporinase.
Ampicillin = 100 for penicillinase.

Classes and sources of β-lactamase were: Ia(1), *Serratia marcescens* 176; Ia(2), *E. cloacae* 91; Ib, *E. coli* 35(C$^+$); Ic, *P. vulgaris* 9; Id, *Pseudomonas aeruginosa* 11(C$^+$); II, *Proteus mirabilis* FP240; III, *E. coli* 18(R$^+$); IV, *Klebsiella pneumoniae* FP239; V, *P. aeruginosa* 200; *B. fragilis*, *B. fragilis* FP764; *S. aureus*, *S. aureus* 35.

Table 3. Antibacterial activities of FK027 and 3 other β-lactam antibiotics against ampicillin-resistant strains of *E. coli*.

Antibiotic	Mean MIC (μg/ml)		
	R$^+$ *E. coli*[a]	C$^+$ *E. coli*[b]	Clinical isolates of *E. coli*
FK027	0.696 (0.1~100)[c]	0.526 (0.2~3.13)	0.628 (0.05~100)
Cefaclor	3.72 (0.78~>100)	7.62 (3.13~25)	3.06 (0.39~>100)
Cephalexin	9.36 (3.13~>100)	11.3 (6.25~25)	10.3 (3.13~>100)
Amoxicillin	>100 (>100)	>100 (>100)	30.1 (0.78~>100)

[a] Ampicillin-resistance plasmid harboring strains (12 clinical isolates).

[b] Chromosomally resistant strains (14 clinical isolates).

[c] Parentheses indicate ranges of MIC distributions.

lity of FK027 to both penicillinases coded by ampicillin-resistance plasmids and chromosomally mediated cephalosporinase of *E. coli*, FK027 was the most active against both types of resistant strains, with mean MICs of 0.70 μg/ml for the plasmid harboring strains and 0.53 μg/ml for the chromosomally resistant strains (Table 3). These mean MICs were close to the mean MIC for the clinical isolates of *E. coli*. Additionally, to evaluate the net effect of ampicillin-resistance plasmids on the antibacterial activity of FK027, MICs of FK027 for *E. coli* CSH2 were compared with those for the strains of *E. coli* CSH2 possessing ampicillin-resistance plasmids. Introduction of the ampicillin-resistance plasmids into the host strain was ascertained scarcely to affect the antibacterial activity of FK027 (Table 4). The antibacterial activity of cephalexin and cefaclor was rather decreased by the chromosomally mediated cephalosporinase, but the reduction in their activity was not so great as expected from their instability to cephalosporinase (Table 3). This may be ascribed to the fine ability of cephalexin and cefaclor to penetrate the outer membrane, because the periplasmic concentrations of β-lactam antibiotics, which are effective on the target enzymes, can be determined synergistically by their stability to β-lactamase and their ability to penetrate the outer membrane[6,15]. Reversely, FK027 seemes to compensate for

Table 4. Influence of introduced ampicillin-resistance plasmids on antibacterial activity of FK027 against *E. coli* CSH 2.

Antibiotic		MIC (μg/ml) distribution												
		0.2	0.39	0.78	1.56	3.13	6.25	12.5	25	50	100	200	400	>400
FK027	Parent				1									
	R^+			3	10									
Cefaclor	Parent								1					
	R^+								4	2	4	3		
Cephalexin	Parent										1			
	R^+									5	2	5	1	
Amoxicillin	Parent							1						
	R^+													13

Over night cultures in Mueller-Hinton broth containing 10^6 cfu were spot-inoculated.

its relatively poor ability to penetrate the outer membrane with its high stability to β-lactamase.

Discussion

The third generation cephalosporins, what is called, are distinct from the older β-lactam antibiotics in their intensive antibacterial activity against a wide range of Gram-negative bacteria. The exceptional antibacterial activity of the third generation cephalosporins has been shown to be based on both their enhanced affinity for the target enzymes and their high stability to the β-lactamases[16]. We have confirmed that FK027 shares similar characteristics with the other third generation cephalosporins. First, we showed that FK027 is extremely stable to the plasmid mediated penicillinases as well as the chromosomally mediated β-lactamases except the β-lactamase of *B. fragilis*. The cephalosporin derivatives containing oxyimino ether groups in the 7-substitute generally show less stability to the cefuroximase classified by MITSUHASHI[17], which includes the chromosomal β-lactamases of *B. fragilis, Proteus vulgaris* and *Pseudomonas cepacia* though the level of stability depends on the derivatives. FK027 belonging to this family possesses the same tendency. The high stability of FK027 to each type of plasmid-mediated penicillinase assures that the antibacterial activity of FK027 is free from the effect of the ampicillin-resistance plasmids. On the other hand, its high stability to the species-specific β-lactamases is supposed to contribute to its antibacterial activity against the corresponding species. The moderate instability of FK027 to the β-lactamase of *B. fragilis* appears to be the cause of its relatively weaker activity against this species. However, this does not constitute a disadvantage of FK027 as an orally absorbed antibiotic due to the following reason. One of the most serious adverse effects of orally absorbed antibiotics is pseudomembranous colitis. It is supposed that this colitis is caused by enterotoxin-producing *Clostridium difficile* which increases under the alterations of intestinal microflora[18]. Accordingly, it is better for oral antibiotics to possess less activity against the major species constituting intestinal microflora such as *B. fragilis*. Second, we showed that the ability of FK027 to penetrate the outer membrane is weaker than that of existing orally absorbed cephalosporins. The carboxyl anion in the 7-substituent of FK027 seems to be the cause of this weaker ability since the ability of a compound to penetrate the outer membrane was reported to be decreased by negative charges[19]. However, the relatively poor ability to penetrate the outer membrane is a common feature of the third generation cephalosporins[16], amongst which ceftizoxime has outstanding ability as we already showed[13]. Finally, FK027 was shown to possess much higher affinity for the PBPs 3, 1a and 1bs than existing orally absorbed cephalosporins. The excellent antibacterial activity of FK027 against Gram-negative bacteria can be explained by this high affinity for target enzymes as well as its high stability to β-lactamases. The affinity of FK027 for the PBPs 1 and 3 of *S. aureus*, two of the essential target enzymes, was less than that of cephalexin. This result suggests that the relatively low affinity of FK027 for PBPs of *S. aureus* is responsible for its less potent antibacterial activity against *S. aureus*.

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