CEPHALOSPORINASE INTERACTIONS AND ANTIMICROBIAL ACTIVITY OF BMY-28142, CEFTAZIDIME AND CEFOTAXIME

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Cephalosporinases of *Enterobacter cloacae* and *Citrobacter freundii* were responsible for resistance to newer cephalosporins such as cefotaxime and ceftazidime but not BMY-28142. Interaction of these cephalosporins including hydrolysis, binding, inhibition, and inactivation with cephalosporinases from *E. cloacae* GN7471 and *C. freundii* GN7391 were studied. BMY-28142 was much more stable against the both enzymes than cephalothin, but more hydrolyzable than cefotaxime and ceftazidime at higher concentration such as 100 μM. Because of low affinity for the enzymes, *i.e.* large *Km* and *Ki*, the calculated hydrolysis rate of BMY-28142 at 0.1 μM was smaller than those of cefotaxime and ceftazidime, that explained the difference in activity against cephalosporinase-producing strains between BMY-28142 and cefotaxime or ceftazidime. The effects of cephalosporinase production on susceptibility of *Escherichia coli omp* mutants were examined using a plasmid having cephalosporinase gene of *C. freundii* GN346. Decrease in susceptibility of *E. coli* by cephalosporinase production was larger in the strain lacking outer membrane proteins (Omp) F and C, and smaller in the strain producing OmpF constitutively.

BMY-28142, a new oxyiminocephalosporin, has excellent activity against both Gram-positive and Gram-negative bacteria[1,2]. Among these organisms, *Enterobacter cloacae* and *Citrobacter freundii*, which produce chromosomal cephalosporinases, are more susceptible to BMY-28142 than to other cephalosporins[3~5]. Recently, the emergence of cephalosporinase hyperproducing strains in these species which show resistance to newer cephalosporins, such as cefotaxime and ceftazidime, was reported[6]. On the resistance mechanism of cephalosporinase-high-producing strains against the newer cephalosporins, the theory of a "non-hydrolytic barrier" has been proposed[7,8]. BMY-28142 shows higher activity against these strains than other cephalosporins[5]. The relation of its low affinity, *i.e.* large *Ki* values, for cephalosporinases and its high activity was reported in comparison with ceftazidime[9]. However, how the low affinity leads less influence of cephalosporinase production on activity is still unclear. On the other hand, the importance of very slow hydrolysis by cephalosporinases was also reported[10]. In this paper, we attempted to clarify why BMY-28142 is so effective and examined kinetically the interaction of drugs with cephalosporinases using cephalothin, ceftazidime and

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cefotaxime in comparison with BMY-28142.

Materials and Methods

Antibiotics

BMY-28142 was synthesized by Bristol-Myers Company in Syracuse, New York, and its sulfate salt (830 μg/mg) was used in this study. Other antibiotics were donated by Shin Nihon Jitsugyo Co., Ltd., for ceftazidime, Hoechst Japan Limited for cefotaxime, Merck-Banyu Co., Ltd., for cefoxitin, Shionogi & Co., Ltd., for cephalothin.

Bacteria and Plasmid

Escherichia coli MC4100 (F⁻ *ΔlacU169 araD139 rpsL relA thiA fibB*)[11], *E. coli* MH1160 (MC4100 *ompR1*)[12], and *E. coli* MH760 (MC4100 *ompR2*)[13] were kindly donated by S. MIZUSHIMA (Nagoya University, Nagoya, Japan). MH1160 lacks outer membrane proteins (Omp) F and C production. MH760 lacks OmpC production and produces OmpF constitutively, while in the parent strain, MC4100, OmpF and OmpC production is osmo-regulated. The other strains were clinical isolates from several hospitals in Japan. Plasmid, pMS185, is a derivative of pACYC184[14] carrying cephalosporinase gene from *C. freundii* GN346 (not published).

Media

Sensitivity Disk agar-N (SDN), *i.e.* modified Mueller-Hinton agar, sensitivity test broth, medium B[15], and nutrient agar with 1% sodium citrate (pH 6.8) were used.

Susceptibility Test

Antibiotic susceptibility was tested by a serial 2-fold agar dilution method in SDN. Approximately 10^4 cfu were inoculated onto agar plates. The MIC was defined as the lowest concentration preventing visual growth of bacteria after incubation for 18~20 hours at 37°C.

Assay of Cephalosporinase

Cephalosporinase was assayed by a spectrophotometric method[16], using a UV-265 spectrophotometer (Shimadzu). An absorbance change greater than 0.001 could be detected using this apparatus. Enzyme activity was determined at 30°C and pH 7.0 in 50 mM phosphate buffer, using cephalothin (100 μM) as substrate, and was expressed in terms of mol hydrolyzed per second (Kat). For the determination of cephalosporinase activity, a crude sonicate was used[16]. *Km* and Vmax values were calculated from Lineweaver-Burk plots using least square method. To detect and determine hydrolysis rates of poor substrates, the concentration of cephalosporinases from *E. cloacae* GN7471 and *C. freundii* GN7391 used in the hydrolysis reaction mixture was 0.27 nKat/ml (*E. cloacae*) and 0.20 nKat/ml (*C. freundii*) for cephalothin and 803 nKat/ml (*E. cloacae*) and 160 nKat/ml (*C. freundii*) for the other cephalosporins.

Determination of Inhibition Constant

Ki values were calculated from initial hydrolysis rates of cephalothin (10~100 μM) with and without a suitable concentration of inhibitor by Lineweaver-Burk plots using least square method. Pre-incubation of enzyme with inhibitor was not done to determine equilibrium constant of enzyme-inhibitor (EI) complex formation, because hydrolysis and inactivation were observed in other experiments.

Purification of Cephalosporinase

The cephalosporinase of *E. cloacae* GN7471 and *C. freundii* GN7391 were prepared by sonication and CM-Sephadex ion-exchange chromatography as previously described[15,17]. Further purification was not attempted, because the main active fraction of each enzyme after CM-Sephadex chromatography showed single band in SDS-PAGE, more than 95% purity, and high specificity (8.0 μKat/mg protein for *E. cloacae*, and 1.2 μKat/mg protein for *C. freundii*) comparable to those of previous preparation[15,17]. Protein was determined by the method of LOWRY *et al.*[18], using bovine serum albumin as the standard.

Determination of Binding to the Cephalosporinase from *E. cloacae* GN7471

The binding of cephalosporins to the cephalosporinase of *E. cloacae* GN7471 at 20 μM of drug determined by a centrifugal ultrafiltration method[19] that has been used for determination of serum protein binding. One volume of drug solution (final 20 μM) in phosphate buffer (50 mM, pH 7.0) were mixed with same volume of various concentrations of the cephalosporinase solution (final 0.27~11 μKat/ml) in the buffer. An enzyme-free ultrafiltrate of the mixture was obtained by centrifugation at $3,000 \times g$ for 20 minutes using a Centricon 10 (Amicon). The drug concentration in the filtrate were determined by an agar-diffusion method using *Bacillus subtilis* ATCC 6633 as the test organism in nutrient agar containing 1% sodium citrate. For the control, the buffer was used instead of enzyme solution. Binding was expressed as binding ratio (B), *i.e.* the ratio of drug in the free state to total drug concentration, calculated from the following equation.

$B\ (\%) = 100 \times (C_0 - C_b)/C_0$, where B was binding ratio, and C_b and C_0 were drug concentration in filtrate of the mixture with (C_b) and without enzyme (C_0). Each preparation of ultrafiltrate was carried out within 30 minutes and below 4°C to minimize hydrolysis of drug, and was performed in duplicate. An enzyme concentration in which each agent showed half binding was obtained from binding-enzyme concentration curve.

Determination of Inactivation against the Cephalosporinase from *C. freundii* GN7391

The cephalosporinase from *C. freundii* GN7391 (97 nKat/ml: approx 2 μM, estimated by protein concentration and MW 38,000) was incubated at 30°C with 2 μM of cephalosporin. Enzyme activity of the mixture was monitored during incubation using cephalothin as substrate, and was expressed in terms of percentage against activity of the mixture incubated without cephalosporin at same incubation time. No competitive inhibition would be expected in the enzyme activity assay, because the concentration of inhibitor in the assay reaction mixture was lowered below the level at which such inhibition would be detected, by 600-fold dilution with substrate solution. The assay was performed in duplicate.

Estimation of Hydrolysis Velocity

Velocity of hydrolysis at 0.1 μM of substrate was calculated from the following Michaelis-Menten equation.

$$v = Vmax/(1 + Km/[S])$$

For cefotaxime, *Km* values were assumed to be equal to *Ki* values. For the calculation of velocities at the cellular level, the amount and MW of the cephalosporinases from *E. cloacae* GN7471 and *C. freundii* GN7391 were taken to be 10^5 molecules per cell[6], and 44,000 for *E. cloacae*[15] and 38,000 for *C. freundii*[17].

Results

Hydrolysis by Cephalosporinases from *E. cloacae* GN7471 and *C. freundii* GN7391

To detect hydrolysis of BMY-28142, ceftazidime and cefotaxime, approximately 3,000 times (for the *E. cloacae* enzyme) or 1,000 times (for the *C. freundii* enzyme) more enzyme than that used in usual hydrolysis tests was used in this experiment. The initial hydrolysis velocities of BMY-28142, ceftazidime and cefotaxime at each series of initial substrate concentration by the enzyme from *E. cloacae* GN7471 is shown in Fig. 1. Hydrolysis rate-substrate concentration curves of the *C. freundii* enzyme were similar to those by the *E. cloacae* enzyme.

Hydrolysis velocity of BMY-28142 and ceftazidime by these enzymes decreased significantly as substrate concentration decreased. No such reduction was seen with cefotaxime for both enzymes. BMY-28142 was more hydrolyzable than cefotaxime and ceftazidime at 100 μM, which is the con-

centration commonly used in hydrolysis test.

Km, Vmax and *Ki* values were shown in Table 1. The values were calculated from two or three experiments using one preparation of each enzyme. Vmax of BMY-28142 was about 6 times greater than that of ceftazidime for both enzymes, and was 15 times, for *E. cloacae* enzyme, or 120 times, for *C. freundii* enzyme, greater than that of cefotaxime.

Furthermore, the *Km* value of BMY-28142 was about 15 times, for *E. cloacae* enzyme, or 30 times, for *C. freundii* enzyme, greater than that of ceftazidime. For cefotaxime, the exact *Km* values could not be obtained, because those values were far smaller than the substrate concentrations used. During the complete hydrolysis of 10 μM of cefotaxime by the enzyme from *E. cloacae* GN7471, the hydrolysis velocity was constant until the substrate was exhausted. The same observation was obtained from complete hydrolysis of 3 μM of cefotaxime by the enzyme from *C. freundii* GN7391. *Ki* of BMY-28142 was nearly one order of magnitude greater than that of ceftazidime and approximately four orders of magnitude greater than those of the other cephalosporins (Table 1).

Binding of Cephalosporins with the Cephalosporinase from *E. cloacae* GN7471

At 20 μM of drug concentration, BMY-28142 showed much less binding ability with the cephalosporinase from *E. cloacae* GN7471 than ceftazidime or cefotaxime did. The enzyme concentration where the drugs showed 50% binding were 11 μKat/ml for BMY-28142, 3.7 μKat/ml for ceftazidime and 1.1 μKat/ml for cefotaxime. These results indicate that the cephalosporinase could decrease the concentration of cephalosporins in free state.

Reversible Inactivation with the Cephalosporinase from *C. freundii* GN7391

When cefotaxime and ceftazidime were mixed with approximately equimolar (2 μM) of the cephalosporinase from *C. freundii* GN7391, they showed inactivation of the enzyme, whereas

Fig. 1. Hydrolysis by the cephalosporinase from *Enterobacter cloacae* GN7471.

Enzyme concentration in the reaction mixture: 803 nKat/ml. Substrates: BMY-28142 (○), ceftazidime (△) and cefotaxime (●).

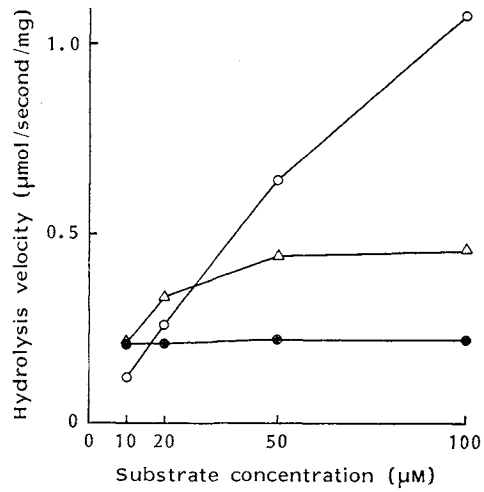


Table 1. Hydrolysis and inhibition kinetic parameters against cephalosporinases from *Enterobacter cloacae* GN7471 and *Citrobacter freundii* GN7391.

Antibiotic	From *E. cloacae* GN7471			From *C. freundii* GN7391		
	Vmax (nmol/second /mg protein)	*Km* (μM)	*Ki* (μM)	Vmax (nmol/second /mg protein)	*Km* (μM)	*Ki* (μM)
Cephalothin	9,300	76		2,100	17	
BMY-28142	3.6	260	>200	5.2	200	>200
Ceftazidime	0.57	17	31	0.85	6.8	37
Cefotaxime	0.22	—	0.08	0.042	—	0.01

—: *Km* values could not be obtained for cefotaxime, because the values were far smaller than the substrate concentrations used.

BMY-28142 did not (Fig. 2). Ceftazidime needed a few minutes of incubation to show maximum inactivation. This inactivation was reversible, and the enzyme activity of the mixture with cefotaxime or ceftazidime was recovered during incubation. Under the same conditions, none of these cephalosporins showed significant inactivation of the cephalosporinase from *E. cloacae* GN7471. These results indicate that the inhibition of cefotaxime and ceftazidime against cephalosporinases of *E. cloacae* and *C. freundii* was not the inactivation such as inactivation by suicide substrates.

Estimation of Hydrolysis Velocity at Low Concentration

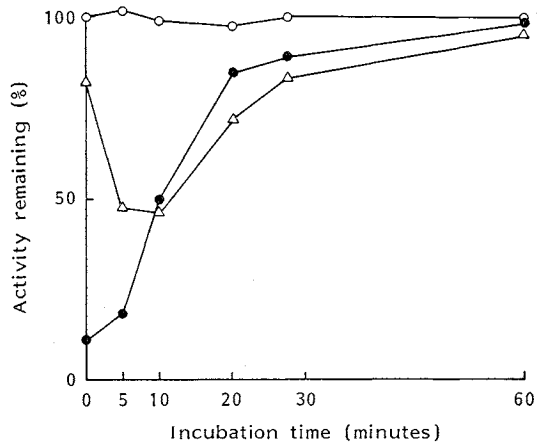
The newer cephalosporins show very low MICs against susceptible strains, and their concentration preventing activity of the targets (penicillin binding proteins (PBPs)) in periplasmic space is supposed as *ca.* 0.1 μM[10]. For comparison of hydrolysis rates of these cephalosporins at cellular level, the velocities at 0.1 μM substrates were estimated from the Michaelis-Menten equation, using Vmax and *Km* (*Ki* for cefotaxime) values (Table 2). At 0.1 μM, the hydrolysis velocity of BMY-28142 was less than 0.07% of Vmax, which was smaller than that of cefotaxime or ceftazidime, while those of cefotaxime and ceftazidime were 54~90% of Vmax and 0.6~1.5% of Vmax, respectively.

Decrease of Antibacterial Activity against *E. coli omp* Mutants by Production of Cephalosporinase of *C. freundii*

The effects of cephalosporinase production on the antibacterial activity of BMY-28142, ceftazidime, and cefotaxime against *E. coli omp* mutants was examined. The cephalosporinase activities of MC4100, MH1160, and MH760 carrying pMS185, which had cephalosporinase gene of *C. freundii* GN346, were 0.67~0.82 nKat/mg protein, whereas those of their host strains were not detected (less than 0.05 nKat/mg protein). Against *E. coli* MC4100, the parental strain, the MIC of cefotaxime or ceftazidime was increased 4 to 8-fold by cephalosporinase production, whereas the MIC of BMY-28142 was unchanged (Table 3). In the case against MH1160, which lacks OmpF and C production, the effects of cephalosporinase were more impressive with

Fig. 2. Reversible inactivation of the cephalosporinase from *Citrobacter freundii* GN7391.

Enzyme (approx 2 μM, 97 nKat/ml) was incubated at 30°C with 2 μM of BMY-28142 (○), ceftazidime (△), or cefotaxime (●).



Enzyme activity of the mixture was monitored during the incubation, and was expressed in the terms of percentage against the activity of the mixture incubated without cephalosporins at same incubation time.

Table 2. Estimation of hydrolysis velocity at 0.1 μM of substrate.

Antibiotic	From *Enterobacter cloacae* GN7471		From *Citrobacter freundii* GN7391	
	nmol/second/mg protein	Molecules/second/cell	nmol/second/mg protein	Molecules/second/cell
Cephalothin	12	54,000	12	45,000
BMY-28142	0.0014	6.0	0.0026	9.5
Ceftazidime	0.0034	15	0.012	46
Cefotaxime	0.12	540	0.038	140

Table 3. Antibacterial activity against *Escherichia coli omp* mutants with or without carrying pMS185 which has cephalosporinase gene of *Citrobacter freundii* GN346.

Strain (phenotype)[a]	pMS185[b]	MIC (μg/ml)		
		BMY-28142	Ceftazidime	Cefotaxime
E. coli MC4100 (wild)	−	0.025	0.05	0.025
	+	0.025	0.20	0.20
	+/− ratio	1	4	8
E. coli MH1160 (F^-, C^-)	−	0.10	0.10	0.10
	+	0.39	1.56	1.56
	+/− ratio	4	16	16
E. coli MH760 (F^+, C^-)	−	0.013	0.025	0.025
	+	0.013	0.10	0.05
	+/− ratio	1	4	2

[a] F^- and C^- mean lacking of OmpF and OmpC, respectively. F^+ means constitutive production of OmpF.

[b] + and − are with and without carrying pMS185 which is a cloned plasmid having cephalosporinase gene of *C. freundii* GN346.

an increase in MIC was 16-fold for cefotaxime and ceftazidime and 4-fold for BMY-28142. Against MH760, which lacks OmpC and produces OmpF constitutively, the increase in MIC of cefotaxime by cephalosporinase production was smaller than MC4100, while that of ceftazidime was as same as in MC4100. The MICs of BMY-28142 against MC4100 and MH760 were not affected by cephalosporinase production. These results indicate that *omp* mutation, which would decrease the outer membrane permeation rate of β-lactams, would increase the effects of cephalosporinase on antimicrobial activity of cephalosporins including BMY-28142.

Discussion

BMY-28142 showed much lower affinity for cephalosporinases (*i.e.* high *Km* and *Ki*) than cefotaxime and ceftazidime. This observation agrees with the finding of PHELPS *et al.*[9], that the excellent antibacterial activity of BMY-28142 originates in its low affinity for cephalosporinases. It is well known that some species of cephalosporinase-producing Gram-negative bacteria, such as *E. cloacae*, are less susceptible to cephalosporinase-stable β-lactams, *e.g.* cefotaxime, ceftazidime, *etc.*[20,21]. As to the mechanism of this resistance, there are controversial theories, in which affinity to cephalosporinase and very slow rate of hydrolysis are both considered[9,21~23].

Hydrolysis reaction of β-lactams by β-lactamases is expressed in the following equation[20].

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES \xrightarrow{k_2} M \xrightarrow{k_3} E + P$$

where E is enzyme (β-lactamase), S is substrate (β-lactam), ES is Michaelis complex, M is acyl intermediate, and P is product (inactivated β-lactam).

We determined the hydrolysis rates of cephalosporinase-stable cephalosporins (Table 1). Their Vmax values were significantly smaller than that of cephalothin. Among these cephalosporins, BMY-28142 was most hydrolyzable at 100 μM. Therefore Vmax of hydrolysis alone cannot explain the activity of BMY-28142. However, the hydrolytic velocity of BMY-28142 decreased as the substrate concentration decreased, whereas that of cefotaxime did not (Fig. 1). To evaluate the role of hydrolysis velocity on the resistance to cephalosporins which are poor substrate, we estimated the rate of hydrolysis at low substrate concentration (0.1 μM). Although cefotaxime and ceftazidime possess inhibitory activity against cephalosporinases, the inhibition was not irreversible inactivation (Fig. 2). Therefore it is reasonable to consider that Michaelis-Menten equation may be applicable to the hydrolysis reaction. Because the hydrolysis rate of cefotaxime did not decrease at the time when the

substrate was almost exhausted, the *Km* of cefotaxime was assumed to be equal to *Ki* in the estimation of hydrolysis rate of cefotaxime. From our estimation, the hydrolysis rate of BMY-28142 at 0.1 μM was much lower than that of cefotaxime, and the difference in hydrolysis rate at 0.1 μM well explained the difference in antimicrobial activity between BMY-28142 and cefotaxime. However, the rate of hydrolysis at 0.1 μM could not explain the difference in activity between BMY-28142 and ceftazidime sufficiently, because the hydrolysis rate of ceftazidime at 0.1 μM was only 3 or 5 times greater than that of BMY-28142.

On the other hand, we observed that presence of cephalosporinase decreased drug concentration in free state by binding. This binding might include both Michaelis complex (ES) and acyl intermediate (M) formation. Against the cephalosporinase from *C. freundii*, ceftazidime and cefotaxime showed reversible inactivation. This might be formation of acyl intermediate (M) which had rather long half-life. "Trapping" or "sponge effects" may be reduction of free drug concentration in periplasmic space by formation of M and/or ES complex which are the parts of hydrolysis reaction.

Because the hydrolysis rates of these cephalosporins was so slow, there needed consideration with permeation through outer membrane. Instead of direct comparison of hydrolysis rate with permeation rate, we determined the effects of cephalosporinase production on antimicrobial activity of the cephalosporins against *E. coli omp* mutants using a cloned plasmid carrying cephalosporinase gene of *C. freundii*. The permeation of cephalosporins through outer membrane has been considered to be simple diffusion through porin channels in outer membrane[24]. *E. coli* MH1160 lacks OmpF and C, so the permeation rate of cephalosporins in MH1160 may be lower than in *E. coli* MC4100 which is a parent of MH1160. Because of constitutive production of OmpF in MH760, the permeation rate of cephalosporins may be rather higher than in MC4100. When these strains produced cephalosporinase of *C. freundii*, susceptibility of these strains against cefotaxime, ceftazidime and BMY-28142 decreased. The effects of the cephalosporinase was most significant in MH1160, in which permeation rate might be lower than in MC4100. Decrease in susceptibility of MH760 against cefotaxime was lower than that in MC4100, while the effect on susceptibility against ceftazidime was at same level in both MH760 and MC4100. Antimicrobial activity of BMY-28142 was affected by the cephalosporinase only against MH1160. These results indicate that ceftazidime had rather lower permeability than cefotaxime or BMY-28142, and also indicate that outer membrane permeability might lead synergic effects with hydrolysis by cephalosporinase on the resistance to these cephalosporins.

On the difference in antimicrobial activity between BMY-28142 and ceftazidime, there are two factors to be considered in addition to hydrolysis velocity. One is permeability through outer membrane discussed above. The other is affinity to PBPs. If ceftazidime needs rather higher concentration to inhibit PBPs than other cephalosporin does as discussed by VU and NIKAIDO[10], hydrolysis rate of ceftazidime may increase proportional to it concentration. Thus the difference in antimicrobial activity between BMY-28142 and ceftazidime may be explained by the synergic effects of the two factors and hydrolysis.

On the characteristics of β-lactams which activity is less affected by cephalosporinase production, there may be at least three types of agents. One is high stability against cephalosporinases at low concentration, that originates low affinity for the enzymes. BMY-28142 possesses this characteristics. Another is high stability caused by high affinity for the enzymes and acyl intermediate formation that have very long half life. Latamoxef may be this type[25]. The other is high permeability through outer membrane which would sufficiently reduce the effect of hydrolysis. Imipenem may be this type of agent[24].

In conclusion, both "non-hydrolytic barrier", *i.e.*, "trapping" or "sponge effects", and "very slow hydrolysis" are the theories in which hydrolysis reaction is watched from each side. Because of low affinity for cephalosporinases, BMY-28142 might be stable against the enzymes at low concentration still sufficient to inhibit the activity of PBPs. Therefore it showed excellent activity for cephalosporinase-producing bacteria.

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