COMBINATION EFFECT OF CLINDAMYCIN WITH CEFTAZIDIME AND CEFOPERAZONE IN INDUCING FILAMENTOUS GROWTH OF *Enterobacter cloacae* AND *Pseudomonas aeruginosa*

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β-Lactam antibiotics are widely used for the treatment of bacterial infections, but some bacteria are known to resist β-lactam antibiotics[1~3], by producing β-lactamase as one mechanism[2,4~9].

Clindamycin (CLDM) inhibits protein synthesis in Gram-positive bacteria (growth-inhibitory effect) but has less or little activity against Gram-negative aerobic bacteria such as *Enterobacter* and *Pseudomonas*[3,10~12]. It was demonstrated that CLDM partially inhibited β-lactamase biosynthesis in *Enterobacteria* species which possess inducible β-lactamase[13]. Further, it has also been demonstrated that CLDM reduced the β-lactamase biosynthesis in *Enterobacteria* and *Pseudomonas* bacteria[14,15]. This information may provide the possibility of combination therapy using β-lactam antibiotics with CLDM against β-lactam antibiotic-resistant bacteria. It is necessary to first clarify the *in vitro* efficacy of β-lactam antibiotics against these bacteria in the presence of CLDM before proposing clinical therapy.

In the present study, we investigated the effect of CLDM with cefoperazone (CPZ) and ceftazidime (CAZ) in inhibiting the growth of *Enterobacter* and *Pseudomonas* bacteria. Some of β-lactam antibiotics, such as CPZ and CAZ, inhibit cross-wall formation during fission by inhibiting an action of penicillin binding protein-3, resulting in filamentous growth[16]. Therefore, we focused this study on the effect of CLDM on CPZ and CAZ in inducing the formation of filamentous bacterial growth.

CLDM hydrochloride was supplied by the Upjohn Company (Michigan, U.S.A.). CPZ, CAZ and sulbactam (SBT) were purchased commercially. Reagents used were of analytical grade. Clinically isolated bacterial strains from sputum used in the present study were *Enterobacter cloacae* (E-11 and E-7) and *Pseudomonas aeruginosa* (P-24 and P-12) obtained from the Respiratory Internal Medicine Unit in Kawasaki Medical School (Okayama, Japan).

The MIC of CAZ or CPZ against the selected bacterial strains was determined by a liquid dilution method using Mueller-Hinton broth and monitoring bacterial growth by densitometry (MIC-2000 Dainihon-Seiki, Japan) according to method reported previously[15].

Bacterial growth was determined by spectrophotometric method reported previously[17]; each strain (about 1×10^8 CFU/ml) was suspended in

Fig. 1. Profiles of medium density after culture of E-11 (A) and P-12 (B).

Key: (A) ●, control (no drug); △, ○, □, CAZ (32, 64 and 128 μg/ml, respectively) alone; ▲, CLDM (20 μg/ml) alone; ▽, combination of CAZ (16 μg/ml) and SBT (8 μg/ml); and ■, combination of CAZ (16 μg/ml) and CLDM (20 μg/ml).

(B) ●, control; △, ○, □, CPZ (16, 32 and 64 μg/ml, respectively) alone; ▲, CLDM (50 μg/ml) alone; ▽, combination of CPZ (16 μg/ml) and SBT (8 μg/ml) and ■, combination of CPZ (16 μg/ml) and CLDM (50 μg/ml).

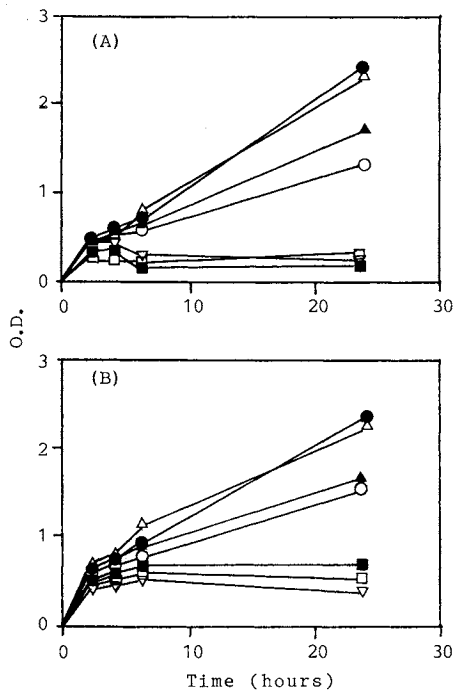
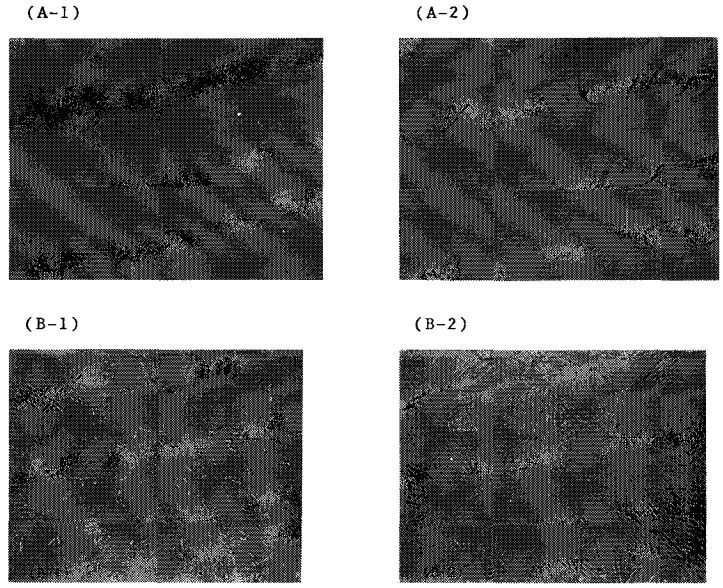


Fig. 2. Filamentous growth of E-11 in the presence of CAZ and that of P-24 in the presence of CPZ.

(A): (A-1) shape of E-11 in the presence of CAZ at 64 μg/ml alone at 4 hours after starting culture; and (A-2) shape of E-11 in the presence of CAZ at 16 μg/ml and CLDM at 20 μg/ml at 1 hour after starting culture.

(B): (B-1) shape of P-24 in the presence of CPZ at 32 μg/ml alone at 4 hours after starting culture; and (B-2) shape of P-24 in the presence of CPZ at 16 μg/ml and CLDM at 50 μg/ml at 2 hours after starting culture.



MH-broth and cultured at 37°C in air. At designated intervals, 3 ml of the broth was collected and its density was determined at 635 nm.

The filamentous growth of bacteria in the presence of β-lactam antibiotics was investigated by a phase microscopy as follows: each strain (about 1×10^8 CFU 1 ml) was suspended in MH-broth containing CPZ or CAZ and CLDM or SBT, and cultured at 37°C in air. At 0.5 hour intervals for 4 hours, 5 μl of the broth was collected and mounted on a glass slide. The filamentous growth of bacteria was determined by a phase microscopy at a magnification of ×2,000. During this study, a total 250 μl of broth was collected at 0, 1, 2 and 4 hours to measure the concentration of CAZ or CPZ in the broth. CAZ or CPZ was assayed by a high performance liquid chromatography[17].

The effect of CAZ (32, 64 and 128 μg/ml) on the growth of E-11 was investigated in the presence or absence of CLDM (20 μg/ml) or SBT (8 μg/ml). As shown in Fig. 1A, CAZ at 32 μg/ml alone had no effect on E-11. CAZ at 64 μg/ml or CLDM at 20 μg/ml alone only retarded the growth of E-11. CAZ at 128 μg/ml alone caused a significant inhibition of E-11 growth. CAZ (16 μg/ml) in combination with CLDM (20 μg/ml) significantly suppressed E-11 growth until 6 hours and killed bacteria by 24 hours. While SBT alone had no influence on the bacteria growth, SBT in combination with CAZ (16 μg/ml) inhibited growth (Fig. 1A) as observed in a combination of CAZ (16 μg/ml) with CLDM.

As summarized in Table 1, E-11 had a rod shape at 4 hours after incubation with CAZ (32 or 64 μg/ml in Fig. 2A), CLDM (20 μg/ml) or SBT (8 μg/ml) alone; *i.e.*, no E-11 filamentous growth occurred. However, with the presence of CLDM (20 μg/ml) in the medium, CAZ even at 16 μg/ml significantly induced filamentous growth at 1 hour (Table 1 and Fig. 2B). This effect was almost the same as that of CAZ alone at 128 μg/ml. The ability of CAZ to induce filamentous growth was also enhanced by SBT (8 μg/ml) present in the MH-broth as seen in Table 1 (CAZ at 16 μg/ml at 1 hour). In the absence of CLDM or SBT, the concentration of CAZ in the broth decreased significantly during the first 4 hours (Table 2). In the presence of SBT, the concentration of CAZ remained unchanged. When CLDM was present in the broth, the concentration of CAZ decreased slightly. The concentration change of CAZ in broth, a parameter of β-lactamase activity,

Table 1. Effect of CLDM or SBT on β-lactam antibiotics (CAZ or CPZ) in inducing formation of filamentous growth of bacteria strains tested during a 4 hours culture.

Strain	Additive in MH-broth (μg/ml)	MIC[a] of CAZ or CPZ μg/ml	Observation of filamentous growth within 4 hours after starting culture[b] (Concentration of CAZ or CPZ in MH-broth, μg/ml)					
			4	8	16	32	64	128
E-11	None	128 (CAZ)				−	−	+(1)
	CLDM (20)	16 (CAZ)		−	+(1)	+(0.5)		
	SBT (8)	16 (CAZ)	−	−	+(1)			
E-7	None	128 (CPZ)				−	−	+(2)
	CLDM (20)	16 (CPZ)		−	+(2)	+(1)		
	SBT (8)	16 (CPZ)		−	+(1)	+(0.5)		
P-24	None	32 (CPZ)			−	−	+(1)	
	CLDM (50)	8 (CPZ)	−	−	+(2)	+(1)		
	SBT	8 (CPZ)	−	+(2)	+(1)			
P-12	None	64 (CPZ)			−	−	+(1)	
	CLDM (50)	16 (CPZ)		−	+(1)	+(0.5)		
	SBT (8)	8 (CPZ)	−	+(2)	+(1)			

[a] MIC of CLDM against four strains tested was more than 256 μg/ml.

[b] −, No filamentous growth was observed during 4 hours; +, filamentous growth was observed within periods described in bracket.

Table 2. Concentration change of CAZ in the broth containing E-11 and CPZ in the broth containing P-24.

Strain	Exp. No.	Concentration of β-lactam antibiotics, μg/ml			
		0 (initial)	1	2	4 (hours)
E-11 (A) in MH-broth containing CAZ alone					
	1	32	28.8±1.2	18.7±1.0	8.4±1.6
	2	64	57.2±2.4	48.1±4.1	21.4±6.9
	3	128	121.3±3.6	110.4±5.2	91.5±9.6
(B) in MH-broth containing CAZ and CLDM (20 μg/ml)					
	4	8	7.2±0.1	6.3±0.3	4.0±0.7
	5	16	15.6±0.2	15.3±0.1	12.4±0.8
	6	32	31.1±0.3	30.4±0.7	28.2±1.2
(C) in MH-broth containing CAZ and SBT (8 μg/ml)					
	7	4	3.9±0.1	3.8±0.1	3.7±0.2
	8	8	7.8±0.2	7.9±0.2	7.7±0.4
	9	16	16.1±0.3	15.8±0.4	15.6±0.2
P-24 (A) in MH-broth containing CPZ alone					
	10	16	14.1±0.6	9.3±1.1	1.9±0.8
	11	32	28.6±1.4	21.2±2.2	9.2±1.9
	12	64	59.8±0.4	54.6±3.1	38.7±4.7
(B) in MH-broth containing CPZ and CLDM (50 μg/ml)					
	13	8	7.9±0.1	7.7±0.1	6.8±0.2
	14	16	15.8±0.2	15.7±0.2	14.5±0.5
	15	32	31.9±0.1	31.6±0.3	30.7±0.2
(C) in MH-broth containing CPZ and SBT (8 μg/ml)					
	16	4	3.9±0.1	3.9±0.3	4.0±0.2
	17	8	8.1±0.2	7.9±0.1	7.7±0.3
	18	16	15.8±0.3	15.9±0.2	15.9±0.2

when compared with the suppression of CAZ degradation by CLDM indicates that CLDM suppressed the biosynthesis of β-lactamase, since it is reported that CLDM does not directly inhibit β-lactamase activity[15].

As shown in Fig. 1B, CPZ (32 μg/ml), CLDM (50 μg/ml), or SBT (8 μg/ml) alone did not inhibit the growth of P-24 at 6 hours, but CLDM slightly retarded the growth at 24 hours. CPZ (16 μg/ml) in combination with CLDM (50 μg/ml) or SBT

(8 μg/ml) significantly inhibited the growth at 24 hours to the extent that CPZ alone did at 64 μg/ml (Fig. 1B). The effect of CLDM or SBT on the ability of CPZ to induce filamentous growth of P-24 strain was also investigated. CPZ (32 μg/ml) did not induce filamentous growth of P-24 even after 4 hours (Table 1). However, when combined with CLDM (50 μg/ml) or SBT (8 μg/ml), CPZ even at 16 μg/ml induced the filamentous bacterial growth (Table 2 and Fig 2B). The concentration of CPZ in MH-broth decreased with the incubation period in the absence of CLDM and SBT (Table 2). In contrast, in the presence of SBT (8 μg/ml), the CPZ concentration remained unchanged, and in the presence of CLDM (50 μg/ml), the CPZ concentration was only slightly decreased (Table 2).

Regarding E-7 and P-12, only the effect of CLDM and SBT on CPZ efficacy in inducing filamentous growth of these bacteria was investigated. As shown in Table 1, CLDM (20 μg/ml or 50 μg/ml) or SBT (8 μg/ml) induced an increase in the CPZ efficacy on filamentous growth of E-7 to some degree, with SBT being somewhat stronger with P-12.

In the present study, the number of bacteria tested was small (only 4 strains), but the concentration of CAZ or CPZ in inducing filamentous growth of each bacteria was decreased by the presence of CLDM or SBT in the broth. We also observed a similar effect in the study of MIC for either CAZ or CPZ (Table 1). MIC of CAZ and CPZ against bacteria in the presence of CLDM or SBT was near to the concentration of CAZ and CPZ inducing filamentous growth by the presence of CLDM or SBT (Table 1). Thus, the filamentous growth by CAZ and CPZ appears to correlate to their inhibitory effect against bacterial growth. Further, it is considered that action of CLDM which only retarded the rate of increase in population of E-11 and P-24 is due to a minor effect of CLDM in suppressing protein synthesis[13,15], since CLDM alone did not induce the filamentous growth of E-11 and P-24 and the MIC of CLDM against both bacteria strains was more than 256 μg/ml.

CAZ and CPZ inhibit cross-wall formation during reproduction by fission by binding to PBP-3[16]. In the β-lactam antibiotic-resistant bacteria, CPZ and CAZ degraded because of β-lactamase decreasing the quantity of these antibiotics[15,18]. As demonstrated in the present study, CLDM, when combined with CPZ and CAZ, enhanced their efficacy in inducing the filamentous growth in the four strains tested (Table 1, and Figs. 1 and 2). SBT is a β-lactamase inhibitor. Consequently in the present study, the concentration of CAZ and CPZ in the broth did not change in the presence of SBT. SBT enhanced the action of CAZ and CPZ on filamentous growth of E-11 and P-24 without causing degradation of these drugs (Table 2). Thus, it was shown that the concentration of CAZ and CPZ that induced filamentous growth of E-11, E-7, P-24, and P-12 was one-eighth when enzymatic degradation of these drugs did not occur. It has been reported that CLDM suppressed the biosynthesis of β-lactamase of some strains of *E. cloacae* and *P. aeruginosa*[13,15], but CLDM did not inhibit β-lactamase activity[18]. Because both CAZ and CPZ hardly degrade in the presence of CLDM in the broth (Table 2), the effect of CPZ and CAZ observed in combination with CLDM were probably due to the inhibitory effect of CLDM on biosynthesis of β-lactamase. In the case of E-11 in broth initially containing CAZ at 64 μg/ml which did not cause filamentous growth, CAZ degraded but the concentration in the medium was higher for 4 hours in comparison to the results obtained with CAZ (16 μg/ml) in the presence of CLDM or SBT which caused the filamentous growth. This may be explained that the target, β-lactamase, was located in the periplasmic space while the concentration of CAZ in the preiplasmic space was significantly smaller than the effective concentration, in spite of CAZ at a high concentration in the medium. A similar explanation may be considered of CPZ against P-24.

As illustrated in Fig. 2, the filamentous growth of bacteria occurred as a result of CAZ and CPZ inhibiting cross-wall formation, *i.e.*, bacterial cell growth, including cell division and cell-wall growth, was continuing while cell separation with individual cell-wall was suppressed due to inhibited cross-wall formation. Thus, it was easily expected that the density of the medium increased in parallel to bacteria cell growth and cell-wall growth even with inhibited cross-wall formation in the first 6 hours (Fig. 1).

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