EFFECT OF COMBINATION OF CEFSULODIN AND β-LACTAM ANTIBIOTICS AGAINST *SERRATIA MARCESCENS*

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(Received for publication July 13, 1981)

The effect of cefsulodin in combination with various β-lactam antibiotics was examined against *Serratia marcescens*. *In vitro*, the optimum ratio for all combinations tested was almost the same (cefsulodin - other antibiotic=1 : 1 ~ 1 : 4). The combinations of cefsulodin-cefazolin and cefsulodin-cefotiam were found to have a synergistic effect and other combinations, such as cefsulodin-cefmenoxime, -ampicillin and -sulbenicillin, an additive effect with the checkerboard dilution and the fixed combination methods. The synergistic effect of cefsulodin-cefotiam was more potent than that of cefsulodin-cefazolin and the effect of both combinations was clearer with heavy than with light inoculum size. With the killing kinetic method, all combinations tested showed a synergistic effect. *In vivo*, the optimum combination ratios of cefsulodin-cefazolin and cefsulodin-cefotiam were 1 : 2 and 1 : 1, respectively, the protective effect of the latter combination being much stronger than that of the former. With the fixed combination method (cefsulodin - other antibiotic=1 : 1 ~ 1 : 4), the effect of the combination of cefsulodin with all antibiotics except cefazolin and cefotiam was additive.

Serratia marcescens is frequently responsible for hospital acquired infections that present serious therapeutic problems[1~5], because of its low susceptibility to many antibiotics and the high frequency of occurrence of strains resistant to most antibiotics. A synergistic effect was reported for the combination of cefsulodin and mecillinam against *S. marcescens*[6]. The present paper examines the effect of combinations of cefsulodin and various β-lactam antibiotics on *S. marcescens in vitro* and *in vivo*.

Materials and Methods

Antibiotics

Cefsulodin, cefotiam, and cefmenoxime were prepared by Takeda Chemical Industries, Ltd., Osaka, Japan. Cefazolin was purchased from Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan. Ampicillin and sulbenicillin were commercial materials from Takeda Chemical Industries, Ltd.

Organisms

The laboratory strains were maintained on Trypticase soy agar (TSA; BBL Microbiology Systems, Cockeysville, Md., USA). Clinical isolates were kindly supplied by several clinical laboratories and maintained on Dorset egg medium (Nissui Seiyaku Co. Ltd., Tokyo).

In Vitro Test

The minimum inhibitory concentration (MIC) was determined by the agar dilution method. The bacteria were cultured overnight at 37°C in Trypticase soy broth (TSB; BBL Microbiology Systems, Cockeysville, Md., USA). One loopful (2 mm in diameter) of a suspension containing about 10^6 and 10^8 colony-forming units (CFU)/ml was streaked for a length of about 2 cm on TSA or MACCONKEY agar (Eiken Chemicals, Tokyo) containing twofold serial dilutions of either or both antibiotics. The MIC was defined as the lowest concentration of antibiotic that prevented visible growth after overnight incubation at 37°C. The effect of the combination was tested by the checkerboard dilution method[7], the fixed combination method[8], and the killing kinetic method[6].

Table 1. Effect of combination of cefsulodin and various β-lactam antibiotics on *in vitro* antibacterial

Combined antibiotic	10^8 CFU/ml[a]								
	IFO 12648			TN 66			TN 73		
	FIC[b]		FIC index	FIC		FIC index	FIC		FIC index
	Cefsulodin	Combined antibiotic		Cefsulodin	Combined antibiotic		Cefsulodin	Combined antibiotic	
Cefazolin	0.25 (100/25)	0.13 (>100/25)	0.38	0.13 (>100/25)	0.13 (>100/25)	0.26	0.25 (>100/25)	0.13 (>100/25)	0.38
Cefotiam	0.03 (100/3.13)	0.03 (>100/6.25)	0.06	0.02 (>100/3.13)	0.06 (50/3.13)	0.08	0.06 (>100/12.5)	0.02 (>100/3.13)	0.08
Cefmenoxime	0.06 (100/6.25)	0.50 (0.1/0.05)	0.56	0.13 (>100/25)	0.50 (0.2/0.1)	0.63	0.25 (>100/50)	0.25 (0.2/0.05)	0.50
Ampicillin	0.25 (100/25)	0.25 (50/12.5)	0.50	0.25 (>100/50)	0.25 (25/6.25)	0.50	ID (>100/>100)	ID (>100/>100)	ID
Sulbenicillin	0.25 (100/25)	0.25 (12.5/3.13)	0.50	0.13 (>100/25)	0.25 (12.5/3.13)	0.38	0.25 (>100/50)	0.25 (12.5/3.13)	0.50

a) Inoculum size was one loopful of bacterial suspension.
b) Fractional Inhibitory Concentration.
c) Number in parentheses indicates MIC of first antibiotic alone/MIC of first antibiotic in combination of

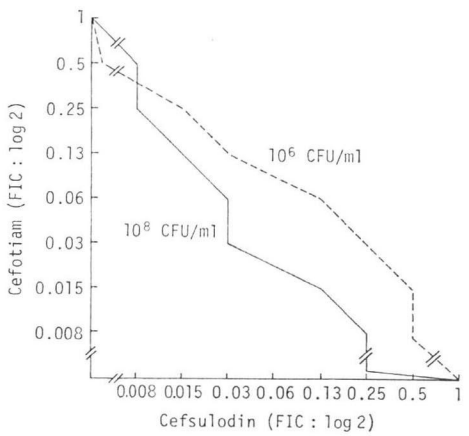
In Vivo Test

Four-week old male Slc: ICR mice, weighing 19~23g, (Shizuoka Agricultural Cooperative Association for Laboratory Animals, Shizuoka, Japan) were used. *S. marcescens* TN 66 was cultured in brain heart infusion (BHI; Difco Laboratories, Detroit, Mich., USA) overnight at 37°C and suspended in 5% mucin (Difco Laboratories, Detroit, Mich., USA). Mice were infected intraperitoneally with 1~3 × 10^8 CFU in 0.5 ml of the bacterial suspension per mouse. The challenge dose was about 100 times the number of organisms required to kill one half of the challenged, non-treated mice. All experiments were repeated at least 5 times. The 50% effective dose (ED_{50}: mg/kg) was calculated by the probit method from the survival rate, recorded 5 days after infection[9]. The effect of the combination was tested by the checkerboard dilution method and the fixed combination method[6].

Criteria for the Combination Effect

To compare the effect of cefsulodin plus other antibiotic with each of the two antibiotics individually the fractional inhibitory concentration (FIC) and FIC index were calculated according to the method reported by ELION *et al.*[10] and to assess the protective effect of the combination of the two antibiotics, the fractional effective dose (FED) and FED index were substituted for the FIC and FIC index[6].

Fig. 1. Isobologram showing synergy of cefsulodin and cefotiam against *S. marcescens* IFO 12648 with the inoculum sizes of 10^8 CFU/ml and 10^6 CFU/ml.



Results

In Vitro Test

Checkerboard-combination

The FIC and FIC index values of the combination of cefsulodin and various β-lactam antibiotics calculated by the checkerboard dilution method on *S. marcescens* IFO 12648, TN 66, and TN 73 are summarized in Table 1 and a typical isobologram showing synergy of cefsulodin and

activity against *S. marcescens* IFO 12648, TN 66, and TN 73 with the checkerboard dilution method.

10^6 CFU/ml								
IFO 12648			TN 66			TN 73		
FIC		FIC index	FIC		FIC index	FIC		FIC index
Cefsulodin	Combined antibiotic		Cefsulodin	Combined antibiotic		Cefsulodin	Combined antibiotic	
0.25 (100/25)	0.25 (>100/12.5)	0.50	0.13 (100/12.5)	0.13 (>100/25)	0.26	0.06 (>100/12.5)	0.13 (>100/25)	0.19
0.03 (100/3.13)	0.13 (6.25/0.78)	0.16	0.25 (>100/25)	0.25 (1.56/0.39)	0.50	0.02 (100/3.13)	0.13 (6.25/0.78)	0.19
0.25 (100/25)	0.25 (0.1/0.025)	0.50	0.50 (100/50)	0.50 (0.1/0.05)	1.00	0.25 (100/25)	0.50 (0.1/0.05)	0.75
0.25 (100/25)	0.25 (50/12.5)	0.50	0.50 (100/50)	0.25 (12.5/3.13)	0.75	ID (>100/>100)	ID (>100/>100)	ID
0.25 (50/12.5)	0.50 (6.25/3.13)	0.75	0.50 (100/50)	0.13 (6.25/0.78)	0.63	0.01 (100/1.56)	0.50 (12.5/6.25)	0.51

second antibiotic.

cefotiam against *S. marcescens* IFO 12648 is shown in Fig. 1. A synergistic effect (FIC index; lower than 0.5) was observed with the combination of cefsulodin-cefazolin and cefsulodin-cefotiam. With an inoculum size of 10^8 CFU/ml, the synergistic effect of cefsulodin-cefotiam was greater than that of cefsulodin-cefazolin. The effect of the combination of cefsulodin and other β-lactam antibiotics was additive.

Fixed-combination

Cefsulodin and various β-lactam antibiotics alone and the fixed combination of cefsulodin and other antibiotics against 36 clinical isolates of *S. marcescens* were examined with inoculum sizes of 10^8 and 10^6 CFU/ml (Table 2). About half of the strains were resistant to cefazolin, cefotiam, ampicillin, and sulbenicillin used alone or in combination with cefsulodin. These strains were classified as indifferent. Cefmenoxime-resistant strains were not observed. With the heavy inoculum, the combination of

Table 2. Effect of the combination of cefulodin and various β-lactam antibiotics on *in vitro* antibacterial activity against clinical isolates of *S. marcescens* with the fixed combination method.

Combined antibiotic	Combination ratio (Cefsulodin *vs.* combined antibiotic)	No. of strains exhibiting;							
		10^8 CFU/ml[a]				10^6 CFU/ml			
		Synergy	Addition	Antagonism	Indifference	Synergy	Addition	Antagonism	Indifference
Cefazolin	1 : 2		13		23	11	5		20
	1 : 4		9		27	2	13		21
Cefotiam	1 : 1	17			19	5	15		16
	1 : 2	16	1		19	7	13		16
Cefmenoxime	1 : 1		31	5			36		
	1 : 2		35	1			35	1	
Ampicillin	1 : 2		18		18		18		18
	1 : 4		18		18		18		18
Sulbenicillin	1 : 2		18		18		11	8	17
	1 : 4	1	17		18		14	5	17

[a] Inoculum size was one loopful of bacterial suspension.

cefsulodin-cefotiam had a synergistic effect and that of cefsulodin and the other β-lactam antibiotics an additive effect. With the light inoculum, the synergistic effect of the combination of cefsulodin-cefazolin was slightly better than that of cefsulodin-cefotiam, and the combination of cefsulodin and the other β-lactam antibiotics showed additive effects as with the heavy inoculum size. Cefmenoxime alone was most active against clinical isolates and all strains were susceptible to this antibiotic.

Killing-kinetic

The bactericidal activity of cefsulodin in combination with various β-lactam antibiotics against *S. marcescens* IFO 12648 was compared with that of the antibiotic alone. The results are summarized in Table 3 and, as a typical picture of the killing kinetic test, the killing curves by cefsulodin and cefotiam against *S. marcescens* IFO 12648 are shown in Fig. 2. The combination of cefsulodin with all β-lactam antibiotics tested showed a synergistic effect.

Table 3. Effect of the combination of cefsulodin and various β-lactam antibiotics on killing kinetics against *S. marcescens* IFO 12648.

Combined antibiotic	Concentration (μg/ml)		Rate of change[a] of No. of CFU	Effect of combination
	Cefsulodin	+ Combined antibiotic		
Cefazolin	12.5	100	5×10^{-6}	Synergy
	25	100	2×10^{-5}	Synergy
Cefotiam	12.5	0.39	2×10^{-4}	Synergy
	25	0.39	1×10^{-4}	Synergy
Cefmenoxime	12.5	0.025	6×10^{-4}	Synergy
	25	0.025	1×10^{-5}	Synergy
Ampicillin	12.5	6.25	3×10^{-5}	Synergy
	25	6.25	4×10^{-6}	Synergy
Sulbenicillin	12.5	1.56	1×10^{-2}	Synergy
	25	1.56	6×10^{-5}	Synergy

[a] The number of CFU by combination of antibiotics/the number of CFU by antibiotic alone.

In Vivo Test

Checkerboard-combination

The ED_{50} values and FED indexes were calculated from the survival rates of mice treated with cefsulodin, cefazolin or their combination (Table 4) and with cefsulodin, cefotiam or their combination (Table 5). The optimal combination ratio for cefsulodin-cefazolin was 1 : 2 and that for cefsulodin-cefotiam was 1 : 1. With the optimal combination ratios, the FED indexes were 0.282 for cefsulodin-cefazolin and 0.438 for cefsulodin-cefotiam, and the ED_{50} values were 47.7 mg/kg for the former combination and 9.71 mg/kg for the latter.

Fig. 2. Killing curves showing synergy of cefsulodin (CFS) and cefotiam (CTM) against *S. marcescens* IFO 12648.

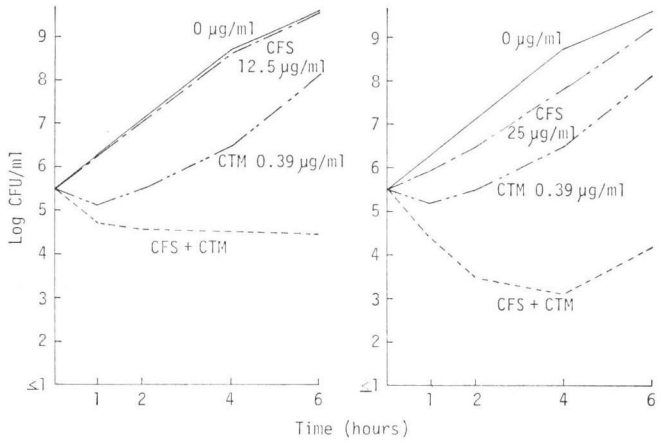


Table 4. Effect of the combination of cefsulodin and cefazolin on intraperitoneal infection with *S. marcescens* TN 66 in mice.[a)]

Ratio			ED_{50}; mg/kg (95% confidence limit)	FED[b)]		FED Index
Cefsulodin		Cefazolin		Cefsulodin	Cefazolin	
Alone		—	113 (96.1~133)			
—		Alone	226 (184~279)			
64	:	1	111 (92.3~133)	0.965 (109/113)	0.008 (1.71/226)	0.973
32	:	1	102 (85.9~119)	0.875 (98.9/113)	0.014 (3.09/226)	0.889
16	:	1	77.1 (64.9~90.8)	0.643 (72.6/113)	0.020 (4.54/226)	0.663
8	:	1	74.8 (63.0~89.5)	0.589 (66.5/113)	0.037 (8.31/226)	0.626
4	:	1	56.7 (46.5~67.9)	0.401 (45.4/113)	0.050 (11.3/226)	0.451
2	:	1	46.0 (39.0~54.6)	0.272 (30.7/113)	0.068 (15.3/226)	0.340
1	:	1	47.4 (39.4~57.0)	0.210 (23.7/113)	0.105 (23.7/226)	0.315
1	:	2	47.7 (39.9~58.3)	0.141 (15.9/113)	0.141 (31.8/226)	0.282
1	:	4	57.7 (43.7~74.2)	0.102 (11.5/113)	0.204 (46.2/226)	0.306

a) Mice were infected intraperitoneally with *S. marcescens* TN 66 (10^3 CFU/mouse) in 0.5 ml of 5% mucin. Antibiotics were administered subcutaneously at 0 hour after infection.

b) Number in parentheses indicates ED_{50} of one antibiotic in the presence of another/ED_{50} of the indicated antibiotic alone.

Table 5. Effect of the combination of cefsulodin and cefotiam on intraperitoneal infection with *S. marcescens* TN 66 in mice.[a)]

Ratio			ED_{50}; mg/kg (95% confidence limit)	FED[b)]		FED Index
Cefsulodin		Cefotiam		Cefsulodin	Cefotiam	
Alone		—	149 (127~176)			
—		Alone	12.0 (9.45~15.2)			
64	:	1	110 (84.1~130)	0.725 (108/149)	0.140 (1.69/12.0)	0.865
32	:	1	66.2 (54.1~80.6)	0.431 (64.2/149)	0.168 (2.00/12.0)	0.599
16	:	1	49.4 (39.9~61.1)	0.312 (46.5/149)	0.242 (2.90/12.0)	0.554
8	:	1	35.4 (27.6~45.2)	0.212 (31.5/149)	0.328 (3.90/12.0)	0.540
4	:	1	22.8 (16.4~31.0)	0.123 (18.2/149)	0.380 (4.60/12.0)	0.503
2	:	1	14.7 (10.6~20.5)	0.066 (9.80/149)	0.409 (4.90/12.0)	0.475
1	:	1	9.71 (6.57~14.0)	0.033 (4.85/149)	0.405 (4.85/12.0)	0.438
1	:	2	11.7 (8.69~15.1)	0.027 (3.90/149)	0.650 (7.80/12.0)	0.683
1	:	4	8.26 (4.93~11.8)	0.011 (1.65/149)	0.551 (6.61/12.0)	0.562
1	:	8	10.2 (5.71~14.2)	0.008 (1.13/149)	0.756 (9.07/12.0)	0.764

a) Mice were infected intraperitoneally with *S. marcescens* TN 66 (10^3 CFU/mouse) in 0.5 ml of 5% mucin. Antibiotics were administered subcutaneously at 0 hour after infection.

b) Number in parentheses indicates ED_{50} of one antibiotic in the presence of another/ED_{50} of the indicated antibiotic alone.

Table 6. Effect of the combination of cefsulodin and various β-lactam antibiotics on intraperitoneal infection with *S. marcescens* TN 66 in mice as determined with the fixed combination method.[a]

Combined antibiotic	ED_{50} (mg/kg)[b]					FED Index		
	Cefsulodin	Combined antibiotic	Cefsulodin+Combined antibiotic			Cefsulodin+Combined antibiotic		
			1 : 1	1 : 2	1 : 4	1 : 1	1 : 2	1 : 4
Cefazolin	141 (115 ~173)	283 (208 ~413)	n.t.[c]	60.1 (48.6 ~74.1)	66.8 (52.4 ~85.8)	n.t.	0.28	0.28
Cefotiam	149 (127 ~176)	12.0 (9.45 ~15.2)	9.71 (6.57 ~14.0)	11.7 (8.69 ~15.1)	n.t.	0.44	0.68	n.t.
Cefmenoxime	115 (95.7 ~137)	0.172 (0.144 ~0.206)	0.329 (0.284 ~0.383)	0.238 (0.196 ~0.288)	n.t.	0.96	0.92	n.t.
Ampicillin	115 (95.7 ~137)	13.0 (10.3 ~16.4)	n.t.	17.1 (13.0 ~22.3)	13.7 (11.0 ~17.2)	n.t.	0.93	0.87
Sulbenicillin	115 (95.7 ~137)	10.2 (7.84 ~13.2)	n.t.	16.4 (13.0 ~20.4)	14.5 (11.6 ~18.2)	n.t.	1.12	1.16

a) Mice were infected with *S. marcescens* TN 66 in 0.5 ml of 5% mucin.

b) Antibiotics were administered subcutaneously at 0 hour after infection. ED_{50} values were calculated by the probit method. Number in parentheses indicates 95% confidence limits.

c) Not tested.

Fixed-combination

The effect of the combination of cefsulodin and various β-lactam antibiotics in ratios of 1: 1, 1: 2, and 1: 4 on mice infected intraperitoneally with *S. marcescens* TN 66 is shown in Table 6. The ratio of combination was chosen referring the dose of each antibiotic used clinically. A synergistic effect (FED index; lower than 0.5) was observed with the combination of cefsulodin-cefazolin and cefsulodin-cefotiam, and an additive effect (FED index; 0.5 ~ 2.0) was observed with the combination of cefsulodin and other β-lactam antibiotics. The protective effect of the combinations reflects the *in vitro* effect of the combinations. The protective effect (ED_{50}) of cefsulodin-cefazolin was the weakest among all combinations tested, and the effect of cefsulodin-cefmenoxime was the best. Other combinations showed similar median protective effects.

Discussion

Over the past decade, *S. marcescens* infections have increased and are now a cause of serious nosocomial infections[1]. The organism is relatively insensitive to most antibiotics. Aminoglycosides have a potent activity against *S. marcescens*[2,3], however, clinical use of aminoglycosides is limited by their serious side effects. Moreover, the number of aminoglycoside-resistant strains of *S. marcescens* has recently increased[4,5]. We have found that the combination of cefsulodin and mecillinam has a synergistic effect against *S. marcescens*[6]. This findings prompted us to investigate more useful combinations between cefsulodin and other β-lactam antibiotics.

Synergistic effects were found with combinations of cefsulodin-cefazolin and cefsulodin-cefotiam, and additive effects were observed with combinations of cefsulodin with cefmenoxime, ampicillin, and sulbenicillin. A synergistic effect is the outcome of one or more steps in a complex series of events, such as, increase of permeability of the antibiotics into the organism, prevention of inactivation of one antibiotic by the other, and inhibition of two sequential steps in a biochemical pathway by the antibiotics[11].

S. marcescens is a constitutive producer of cephalosporinase (RICHMOND's type Ia)[12,13]. Cefazolin and cefotiam are susceptible to hydrolysis by this enzyme[13,14] and other antibiotics are resistant to it[12,15,16]. However, because the affinity of cefsulodin for various β-lactamases is remarkably low[16], a synergistic effect of the combination of cefsulodin-cefazolin or cefsulodin-cefotiam would not be due to the inhibition of *S. marcescens* β-lactamase by cefsulodin.

The optimum ratios of cefsulodin-cefazolin and cefsulodin-cefotiam were almost the same in the two test systems: the *in vitro* susceptibility test and the protective test in mice; but the optimum ratio of cefsulodin-mecillinam was markedly different in the two test systems. These results suggest that the effect of a combination must be tested not only *in vitro* but also *in vivo*.

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