EFFECT OF COMBINATION OF CEFSULODIN AND MECILLINAM

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The effect of cefsulodin in combination with mecillinam was examined against a wide range of bacterial species. The antibacterial spectrum was widened by the combination of cefsulodin and mecillinam in the ratio of 5: 1 and 10: 1. In overall observations, in the *in vitro* test, a synergistic effect against clinical isolates was found on *Klebsiella pneumoniae*, *Citrobacter freundii*, *Enterobacter cloacae*, *Serratia marcescens*, *Proteus mirabilis* and *Proteus vulgaris*, and an additive effect was found on *Staphylococcus aureus*, *Escherichia coli*, *Proteus morganii*, *Proteus rettgeri*, *Proteus inconstans* and *Pseudomonas aeruginosa*. In *in vivo* tests, a synergistic effect was observed on *S. marcescens* TN 66 and *K. pneumoniae* DT infections and an additive effect was observed on *S. aureus* 308 A-1, *E. coli* O-111 and T-7, *C. freundii* TN 518, *E. cloacae* TN 603, *P. vulgaris* GN 4712, *P. morganii* TN 373 and *P. aeruginosa* U 31 infections.

Expansion of the antibacterial spectrum, prevention of superinfection, and enhancement of antibacterial activity in the treatment of overwhelming infections can be expected by use of a combination of antimicrobial agents. Enhancement of antibacterial activity may also result in a reduction in the dose required for therapy and a reduction in the incidence and the severity of adverse reactions.

Cefsulodin and mecillinam have unique antibacterial spectra. The former, a cephalosporin, is active against *Pseudomonas aeruginsa* and some Gram-positive cocci[1~3] and the latter, a penicillin, is active against *Escherichia coli* and *Klebsiella pneumoniae*[4~7]. The two antibiotics have a different mode of antibacterial mechanism. Cefsulodin binds to penicillin-binding protein (PBP) 3 in *P. aeruginosa*[8] and mecillinam binds to PBP 2 in *E. coli*[9]. Treatment of *P. aeruginosa* infections by cefsulodin has produced a high incidence of eradication of *P. aeruginosa* in various clinical specimens from patients, but in some cases, other bacterial species such as *Serratia marcescens, Enterobacter cloacae* and *Proteus* sp. make an appearance in place of *P. aeruginosa*[10]. Mecillinam has been used in the therapy of various kinds of urinary tract infections caused by mecillinam-susceptible organisms[11,12].

These findings suggest that studies on the effect of a combination of cefsulodin and mecillinam would be of interest. The present paper examines the combination of cefsulodin and mecillinam on *in vitro* and *in vivo* activities against a number of Gram-positive and Gram-negative bacteria.

Materials and Methods

Antibiotics

Cefsulodin and mecillinam were prepared in Takeda Chemical Industries, Ltd., Osaka, Japan.

Organisms

The laboratory strains were maintained on Trypticase soy agar (TSA; BBL Microbiology Systems, Cockeysville, Md., U.S.A.). 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, Co-

ckeysville, Md., U.S.A.). 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 of the two antibiotics was tested by the checkerboard dilution method[13] and the fixed combination method[14]. In the checkerboard dilution method, each test consisted of 80 to 150 plates arranged horizontally and vertically, each containing different concentrations of the cefsulodin-mecillinam combination or each antibiotic alone. Eight to 10 rows in one direction contained twofold serial dilutions of cefsulodin, and 10~15 rows in the other direction contained twofold serial dilutions of mecillinam. Two additional rows contained twofold serial dilutions of cefsulodin or mecillinam only. In the fixed combination method, cefsulodin and mecillinam were combined in the ratio of 5: 1 and 10: 1 and the mixture of the two antibiotics was twofold serially diluted. The ratio of the two antibiotics was determined by the doses of both antibiotics used clinically. Killing kinetic studies were also performed. An overnight culture of each bacteria in TSB was inoculated into *fresh* TSB to yield a bacterial concentration of about 10^4 CFU/ml, and the broth was incubated with shaking at 37°C for 1.5~2 hours. A 9 ml portion of the culture, which reached about 10^5 CFU/ml of bacterial concentration, was divided into each flask. A dose of 0.5 ml of each antibiotic solution, prepared in the appropriate concentration, was added to the culture and when the activity of a single antibiotic was determined, 0.5 ml of distilled water was added as a substitute for the second antibiotic. Aliquots of the culture were removed at various time intervals and the number of CFU was determined by the plate count method[15].

In Vivo Test

Four-week old male Slc: ICR mice, weighing 19~23 g, (Shizuoka Agricultural Cooperative Association for Laboratory Animals, Shizuoka, Japan) were used. Organisms were cultured in brain heart infusion (BHI; Difco Laboratories, Detroit, Mich., U.S.A.) overnight at 37°C and suspended in 5% mucin (Difco Laboratories, Detroit, Mich., U.S.A.). Mice were infected intraperitoneally with 0.5 ml of the bacterial suspension. The challenge dose for each infection was about 30~100 times the number of organisms required to kill one half of the challenged, non-treated mice. In the protection test, groups of five mice for each dose level were given 0.2 ml of antibiotic solution subcutaneously immediately after infection. 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[16]. The effect of the combination of cefsulodin and mecillinam was tested by the checkerboard dilution method and the fixed combination method at the combination ratios of cefsulodin to mecillinam of 5: 1 and 10: 1. The number of colony-forming units (CFU) in the peritoneal washing and blood was examined in mice infected with *S. marcescens* TN 66. Blood was collected from the axillary vein and artery under ether anesthesia at various times after infection. After bleeding, 2 ml of TSB was injected into the peritoneal cavity, the external abdominal wall gently massaged for 20~30 seconds and the peritoneal washing collected. The blood and peritoneal washing were serially 10-fold diluted with distilled water and 0.1 ml of the sample was inoculated onto a TSA plate. The plates were incubated overnight at 37°C and the colonies were counted. Bacterial counts were expressed as the number of CFU per ml of the blood and peritoneal washing.

Criteria for the Combination Effect

To compare the effect of cefsulodin plus mecillinam with the two antibiotics individually the fractional inhibitory concentration (FIC) and FIC index were calculated according to the method reported by Elion *et al.*[17] Criteria for the effect of combination were defined as follows: 1) Synergy—the FIC index is lower than 0.5. 2) Addition—the FIC index is 0.5~2.0. 3) Antagonism—the FIC index is higher than 2.0. 4) Indifferent—with the checkerboard dilution method, the MIC of either antibiotic is more than 100 μg/ml and the MIC value of the other is not reduced by the combination. In the fixed combination method, the MICs of both antibiotics are more than 100 μg/ml. For the calculation of the FIC value, when the growth of an organism is not inhibited at a concentration of 100 μg/ml of either antibiotic, the MIC is assessed as 200 μg/ml. On the killing kinetics, the following definitions were

used for the combination effect[18]. 1) Synergy—the CFU declines 100 times or more from that occurring with either antibiotic alone. 2) Addition—the CFU is reduced to one-tenth or less than that occurring with either antibiotic alone. 3) Antagonism—the CFU increases 10 times or more than that occurring with either antibiotic alone. 4) Indifferent—the CFU is within 10 times that produced by either antibiotic alone. To assess the protective effect of the combination of the two antibiotics, the fractional effective dose (FED) and FED index were used as a substitute for the FIC and FIC index.

Results

In Vitro Test

Antibacterial Spectrum

Cefsulodin showed potent antibacterial activity against *S. aureus* and *P. aeruginosa*. The antibacterial activity of mecillinam was potent against *E. coli, K. pneumoniae* and *Serratia marcescens*. By the combination of cefsulodin and mecillinam in the ratio of 5: 1 and 10: 1, the antibacterial spectrum was wider than that of each antibiotic alone and this was more obvious with the inoculum size of 10^8 CFU/ml (Table 1). The mixture of both antibiotics was active against *Citrobacter freundii, Enterobacter cloacae* and *Proteus* sp. which were not affected by each antibiotic alone.

Checkerboard-combination

The FIC and FIC index values of the combination of cefsulodin and mecillinam using the checkerboard dilution method on 26 strains of 14 bacterial species are summarized in Table 2 and a typical isobolograms showing synergy of these antibiotics against *S. marcescens* IFO 12648 and TN 66 are shown in Fig. 1. A synergistic effect (FIC index; lower than 0.5) was observed on both strains of *C. freundii, E. cloacae* and *S. marcescens*, and one of two strains of *Proteus mirabilis, Proteus vulgaris* and *Proteus inconstans*. In particular, the MIC values of cefsulodin and mecillinam against all strains of *C. freundii, E. cloacae* and *S. marcescens* decreased 16~64 times with the combination of both antibiotics. An additive antibacterial effect (FIC index; 0.5~2.0) was observed on 17 out of 27 strains tested. An

Table 1. Antibacterial spectrum of cefsulodin, mecillinam and a mixture of the two antibiotics.

Organism	10^8 CFU/ml[a]				10^6 CFU/ml			
	Cef-sulodin	Mecil-linam	Cef-sulodin + Mecil-linam		Cef-sulodin	Mecil-linam	Cef-sulodin + Mecil-linam	
			5 : 1	10 : 1			5 : 1	10 : 1
Staphylococcus aureus FDA 209P	6.25	25	6.25	6.25	3.13	12.5	3.13	3.13
〃 308 A-1	3.13	25	3.13	3.13	3.13	12.5	3.13	3.13
Escherichia coli NIHJ JC-2	50	0.2	1.56	3.13	50	0.2	0.78	1.56
〃 O-111	50	0.1	0.78	0.78	50	0.05	0.39	0.39
Citrobacter freundii IFO 12681	50	>100	6.25	6.25	50	100	3.13	1.56
Klebsiella pneumoniae DT	50	50	6.25	6.25	50	0.1	0.78	0.78
Enterobacter cloacae IFO 12937	>100	>100	25	25	>100	25	3.13	6.25
Serratia marcescens IFO 12648	100	>100	12.5	12.5	100	1.56	6.25	6.25
Proteus mirabilis IFO 3849	100	100	25	25	100	25	6.25	12.5
Proteus vulgaris IFO 3988	100	>100	25	25	100	50	12.5	12.5
Proteus morganii IFO 3168	>100	>100	100	50	>100	>100	50	50
Pseudomonas aeruginosa U 31	12.5	>100	12.5	12.5	1.56	>100	3.13	3.13

[a] MIC (μg/ml) was determined on TSA. Inoculum size was one loopful of bacterial suspension.

Table 2. Effect of combination of cefsulodin and mecillinam on *in vitro* antibacterial activity with the checkerboard dilution method.

Organism	10^5 CFU/ml[a]			10^6 CFU/ml		
	FIC[b]		FIC index	FIC		FIC index
	Cefsulodin	Mecillinam		Cefsulodin	Mecillinam	
S. aureus FDA 209P	0.25 (3.13/ 0.78)[c]	0.25 (25 / 6.25)	0.50	0.13 (3.13/ 0.39)	0.50 (12.5 / 6.25)	0.63
S. aureus 308 A-1	0.13 (3.13/ 0.39)	0.50 (25 / 12.5)	0.63	0.25 (3.13/ 0.78)	0.25 (25 / 6.25)	0.50
E. coli NIHJ JC-2	0.03 (100 / 3.13)	0.50 (0.2 / 0.1)	0.53	0.25 (100 /25)	0.25 (0.2 / 0.05)	0.50
E. coli O-111	0.25 (50 / 12.5)	0.50 (0.05/ 0.025)	0.75	0.25 (50 /12.5)	0.25 (0.05/ 0.013)	0.50
C. freundii IFO 12681	0.13 (50 / 6.25)	0.001 (>100 / 0.1)	0.131	0.031 (50 / 1.56)	0.001 (100 / 0.1)	0.032
C. freundii TN 518	0.031 (100 / 3.13)	0.001 (>100 / 0.1)	0.032	0.06 (50 / 3.13)	0.25 (0.2 / 0.05)	0.31
S. typhi Boxhill-58	0.50 (25 / 12.5)	0.13 (0.05/ 0.006)	0.63	0.25 (25 / 6.25)	0.13 (0.05/ 0.006)	0.38
S. sonnei EW-33	0.13 (50 / 6.25)	0.13 (0.2 / 0.025)	0.26	0.06 (50 / 3.13)	0.25 (0.1 / 0.025)	0.31
K. pneumoniae IFO 3512	0.063 (100 / 6.25)	0.001 (>100 / 0.05)	0.064	0.031 (50 / 1.56)	0.001 (100 / 0.05)	0.032
K. pneumoniae DT	0.06 (50 / 3.13)	0.25 (0.2 / 0.05)	0.31	0.25 (50 /12.5)	0.13 (0.1 / 0.013)	0.38
E. cloacae IFO 12937	0.13 (>100 /25)	0.008 (>100 / 1.56)	0.138	0.008 (>100 / 1.56)	0.031 (25 / 0.78)	0.039
E. cloacae TN 603	0.031 (100 / 3.13)	0.031 (50 / 1.56)	0.062	0.13 (100 /12.5)	0.13 (1.56/ 0.2)	0.26
S. marcescens IFO 12648	0.062 (100 / 6.25)	0.031 (>100 / 6.25)	0.093	0.25 (50 /12.5)	0.25 (1.56/ 0.39)	0.50
S. marcescens TN 66	0.062 (>100 / 12.5)	0.008 (>100 / 1.56)	0.070	0.062 (100 / 6.25)	0.008 (>100 / 1.56)	0.070
P. mirabilis IFO 3849	0.25 (100 / 25)	0.004 (>100 / 0.78)	0.254	0.062 (100 / 6.25)	0.031 (50 / 1.56)	0.093
P. mirabilis GN 4336	0.25 (50 / 12.5)	0.25 (>100 / 50)	0.50	0.25 (50 /12.5)	0.25 (0.78/ 0.2)	0.50
P. vulgaris IFO 3988	0.25 (100 / 25)	0.13 (>100 / 25)	0.38	0.13 (100 /12.5)	0.002 (>100 / 0.39)	0.132
P. vulgaris GN 4421	0.50 (>100 /100)	0.25 (>100 / 50)	0.75	0.13 (100 /12.5)	0.062 (>100 / 12.5)	0.192
P. morganii IFO 3168	0.50 (>100 /100)	0.001 (>100 / 0.1)	0.501	0.50 (100 /50)	0.001 (>100 / 0.1)	0.501
P. morganii TN 373	0.50 (>100 /100)	0.004 (>100 / 0.78)	0.504	0.50 (100 /50)	0.004 (>100 / 0.78)	0.504
P. rettgeri TN 338	0.50 (>100 /100)	0.50 (>100 / 100)	1.00	0.25 (25 / 6.25)	0.25 (>100 / 50)	0.50
P. rettgeri TN 350	0.50 (50 / 25)	0.25 (>100 / 50)	0.75	0.25 (25 / 6.25)	0.25 (50 / 12.5)	0.50
P. inconstans TN 1683	0.062 (100 / 6.25)	0.001 (>100 / 0.39)	0.063	0.13 (12.5 / 1.56)	0.25 (50 / 12.5)	0.38
P. inconstans TN 1684	0.50 (100 / 50)	0.015 (>100 / 3.13)	0.515	0.25 (25 / 6.25)	0.008 (>100 / 1.56)	0.258
P. aeruginosa U 31	1.00 (12.5 / 12.5)	ID (>100 />100)	ID	1.00 (6.25/ 6.25)	ID (>100 />100)	ID
P. *aeruginosa* GN 3345	0.50 (50 / 25)	0.001 (>100 / 0.2)	0.501	0.50 (12.5 / 6.25)	0.50 (>100 / 100)	1.00

[a] Inoculum size: One loopful of bacterial suspension.

[b] Fractional Inhibitory Concentration.

[c] Number in parentheses indicates MIC of first cephalosporin alone/MIC of first cephalosporin in combination of second cephalosporin.

Fig. 1. Isoborogram showing synergy of cefsulodin and mecillinam against *S. marcescens* with the inoculum sizes of 10^8 CFU/ml and 10^6 CFU/ml.

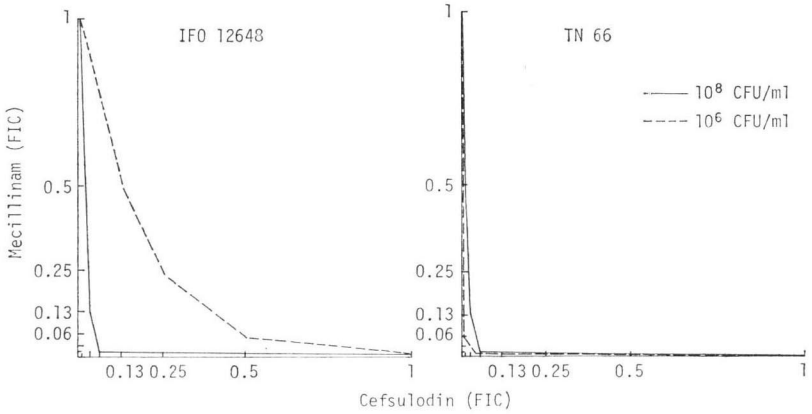


Table 3. Effect of combination of cefsulodin and mecillinam against clinical isolates with the fixed combination method.

Organism (No. of strains tested)	Combination ratio (Cefsulodin *vs.* mecillinam)	No. of strains exhibiting							
		10^8 CFU/ml[a]				10^6 CFU/ml			
		Synergy	Addition	Antagonism	Indifference	Synergy	Addition	Antagonism	Indifference
S. aureus (38)	5 : 1		38				38		
	10 : 1		38			1	37		
E. coli (37)	5 : 1	35	2			6	31		
	10 : 1	34	3			6	31		
C. freundii (38)	5 : 1	32	1		5	7	29		2
	10 : 1	30	3		5	12	23		3
K. pneumoniae (37)	5 : 1	30	6		1	19	18		1
	10 : 1	32	4		1	19	17	1	
E. cloacae (38)	5 : 1	32	2		4	13	24		1
	10 : 1	31	3		4	15	21		2
S. marcescens (36)	5 : 1	27	2		7	31	2		3
	10 : 1	27	2		7	29	2		5
P. mirabilis (38)	5 : 1	32	1		5	33	5		
	10 : 1	34			4	37	1		
P. vulgaris (38)	5 : 1	20	2		16	22	13	1	2
	10 : 1	23	2		13	22	13	1	2
P. morganii (37)	5 : 1	18	19			17	18	2	
	10 : 1	18	17	2		16	20	1	
P. rettgeri (38)	5 : 1	18	18		2	21	15		2
	10 : 1	18	18		2	21	15		2
P. inconstans (27)	5 : 1	21	4		2	17	10		
	10 : 1	21	4		2	18	9		
P. aeruginosa (37)	5 : 1	1	36				37		
	10 : 1		37			5	32		

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

antagonistic effect (FIC index; more than 2.0) was not observed on any of the bacterial strains tested. With the combination of cefsulodin and mecillinam, and mecillinam alone, *P. aeruginosa* U 31 was not inhibited at a concentration of 100 μg/ml: this strain was classified as indifferent.

Fixed-combination

Cefsulodin and mecillinam alone and the fixed combination of the two antibiotics in the ratio of 5: 1 and 10: 1 against clinical isolates were examined and the combined effects are summarized in Table 3. With the inoculum size of 10^8 CFU/ml, a synergistic effect was observed on most strains of *E. coli, C. freundii, K. pneumoniae, E. cloacae, S. marcescens, P. mirabilis, P. vulgaris, P. inconstans* and on about one half of the strains of *P. morganii* and *P. rettgeri*. With the inoculum size of 10^6 CFU/ml, the synergistic effect was observed on most strains of *S. marcescens, P. mirabilis, P. vulgaris,* and *P. inconstans*, and on about one half of the strains of *K. pneumoniae, E. cloacae,* and *P. morganii*. An antagonistic effect was observed on only a few strains of *K. pneumoniae, P. vulgaris*, and *P. morganii*. An indifferent effect was observed on some strains of *C. freundii, K. pneumoniae, E. cloacae, S. marcescens, P. mirabilis, P. vulgaris, P. rettgeri,* and *P. inconstans*. On the remaining strains of various bacterial species, especially on almost all strains of *S. aureus* and *P. aeruginosa*, the effect of combination of cefsulodin and mecillinam was additional.

Killing Kinetic

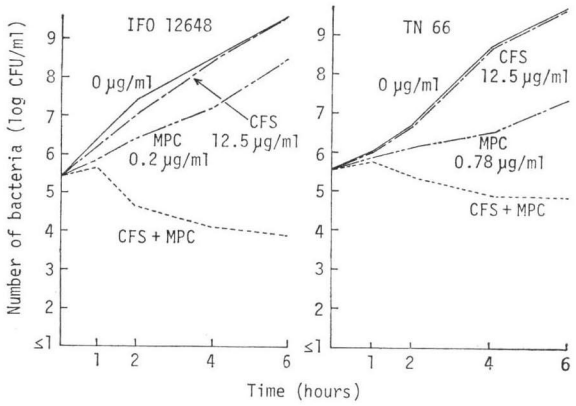
The bactericidal activity of the combination of cefsulodin and mecillinam compared with that of either of the antibiotics is summarized in Table 4 and as a typical picture of the killing kinetics test, the killing curves of the two antibiotics against *S. marcescens* IFO 12648 and TN 66 are shown in Fig. 2. A synergistic effect was observed on *E. coli* NIHJ JC-2, *C. freundii* TN 518, *K. pneumoniae* DT, *E. cloacae* IFO 12937, *S. marcescens* IFO 12648 and TN 66, *P. mirabilis* IFO 3849 and *P. vulgaris* IFO 3988. The number of CFU of these strains was not decreased at the concentrations of each antibiotic used alone and

Table 4. Effect of combination of cefsulodin and mecillinam against 11 strains of bacteria with the killing kinetic method.

Organism	Antibiotic (μg/ml)		Rate of change[a] of No. of CFU	Effect of combination
	Cefsulodin +	Mecillinam		
S. aureus FDA 209P	0.78	6.25	2×10^{-2}	Synergy
	1.56	6.25	2×10^{-4}	Synergy
E. coli NIHJ JC-2	12.5	0.1	8×10^{-4}	Synergy
	25	0.1	3×10^{-6}	Synergy
C. freundii TN 518	12.5	0.1	9×10^{-5}	Synergy
	25	0.1	8×10^{-5}	Synergy
K. pneumoniae DT	12.5	0.05	6×10^{-4}	Synergy
	25	0.05	2×10^{-3}	Synergy
E. cloacae IFO 12937	12.5	0.39	2×10^{-4}	Synergy
	25	0.39	2×10^{-4}	Synergy
S. marcescens IFO 12648	12.5	0.2	3×10^{-5}	Synergy
	25	0.2	2×10^{-5}	Synergy
S. marcescens TN 66	12.5	0.78	3×10^{-3}	Synergy
	25	0.78	3×10^{-3}	Synergy
P. mirabilis IFO 3849	12.5	1.56	6×10^{-4}	Synergy
	25	1.56	2×10^{-4}	Synergy
P. vulgaris IFO 3988	12.5	0.2	7×10^{-3}	Synergy
	25	0.2	6×10^{-5}	Synergy
P. morganii TN 373	12.5	6.25	4×10^{-1}	Addition
	25	6.25	4×10^{-1}	Addition
P. aeruginosa U 31	1.56	400	2×10^{-1}	Addition
	3.13	400	1×10^{1}	Indifferent

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

Fig. 2. Killing curve showing synergy of cefsulodin (CFS) and mecillinam (MPC) against *S. marcescens*.



decreased more than 100 times with the combination of cefsulodin and mecillinam. The effect of the combination against *P. morganii* TN 373 and *P. aeruginosa* U 31 was classified as additional or indifferent, since the number of CFU of these strains was slightly decreased or increased with the combination of both antibiotics compared with either antibiotic alone.

In Vivo Test

Synergistic Effect against *S. marcescens* TN 66

In *in vitro* studies, cefsulodin and mecillinam showed a marked synergistic effect against several laboratory and clinical strains of *S. marcescens*. *S. marcescens* TN 66 was selected as the *in vivo* test organism, since the strain shows potent virulence on intraperitoneal infection with mucin. A synergistic effect of the combination of cefsulodin and mecillinam was observed in the protective test in mice using the checkerboard dilution method. The survival rates of mice treated with either or both antibiotics are shown in Table 5 and the ED_{50} values and FED index calculated from the results presented in Table 5 are

Table 5. Survival rate of mice infected with *S. marcescens* TN 66 treated by a combination of cefsulodin plus mecillinam.[a)]

Mecillinam (mg/kg)	Cefsulodin (mg/kg)						
	0	6.25	12.5	25	50	100	200
0	0/25[b)]	0/25	0/25	0/25	0/25	10/25	20/25
1.56			0/25	0/25	4/25	22/25	25/25
3.13			0/25	3/25	4/25	22/25	25/25
6.25			0/25	2/25	6/25	20/25	25/25
12.5			0/25	4/25	12/25	25/25	25/25
25	0/25	0/25	3/25	6/25	11/25	25/25	25/25
50	0/25	0/25	7/25	10/25	11/25	25/25	25/25
100	0/25	0/25	6/25	10/25	16/25	25/25	25/25
200	4/25	5/25	10/25	14/25	14/25	25/25	25/25
400	8/25	10/25	15/25	22/25	22/25	25/25	25/25
800	15/25	20/25	24/25	25/25	25/25	25/25	25/25

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) No. of survival mice/No. of total mice.

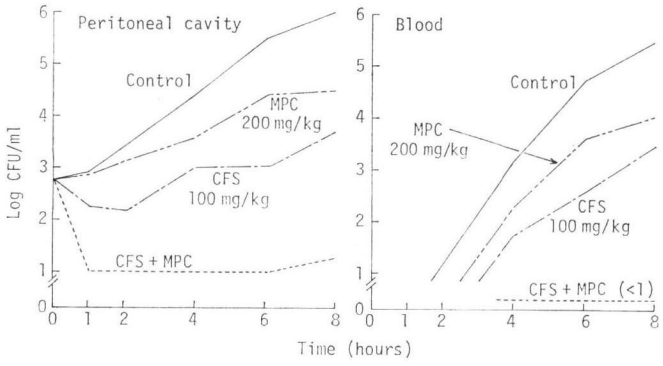
Table 6. Effect of combination of cefsulodin and mecillinam on mice infected with *S. marcescens* TN 66.[a)]

Ratio			ED_{50}; mg/kg (95 % confidence limit)	FED[b)]		FED Index
Cefsulodin		Mecillinam		Cefsulodin	Mecillinam	
Alone		—	124 (103 ~ 149)			
—		Alone	613 (475 ~1,000)			
32	:	1	71.6 (60.9~ 83.6)	0.560 (69.4/124)	0.004 (2.2/613)	0.564
16	:	1	77.2 (65.4~ 91.1)	0.586 (72.7/124)	0.007 (4.5/613)	0.593
8	:	1	65.9 (57.7~ 76.5)	0.473 (58.6/124)	0.012 (7.3/613)	0.485
4	:	1	59.6 (50.4~ 70.3)	0.385 (47.4/124)	0.019 (11.9/613)	0.404
2	:	1	69.1 (57.1~ 83.0)	0.372 (46.1/124)	0.038 (23.0/613)	0.410
1	:	1	88.7 (70.2~ 110)	0.358 (44.4/124)	0.072 (44.4/613)	0.430
1	:	2	94.1 (74.4~ 118)	0.253 (31.4/124)	0.102 (62.7/613)	0.355
1	:	4	150 (60.3~ 444)	0.242 (30.0/124)	0.196 (120 /613)	0.438
1	:	8	199 (162 ~ 245)	0.177 (22.0/124)	0.289 (177 /613)	0.466
1	:	16	250 (210 ~ 297)	0.121 (15.0/124)	0.383 (235 /613)	0.504
1	:	32	332 (272 ~ 399)	0.081 (10.0/124)	0.525 (322 /613)	0.606

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.

Fig. 3. Killing curve showing synergy of cefsulodin and mecillinam on bacterial count in peritoneal washing and blood of mice infected with *S. marcescens* TN 66.



summarized in Table 6. The ED_{50} values of cefsulodin and mecillinam alone were 124 and 613 mg/kg, respectively, and with the combination of the two antibiotics, the ED_{50} values definitely decreased. The smallest ED_{50} value was observed with the ratio of cefsulodin to mecillinam of 4: 1, but the minimum FED index, 0.355, was observed with the ratio of cefsulodin to mecillinam of 1: 2. The number of CFU

Table 7. Effect of combination of cefsulodin and mecillinam on intraperitoneal infection in mice with the fixed-combination method.[a)]

Organism (CFU/mouse)	ED_{50} (mg/kg)[b)]				FED Index	
	Cefsulodin	Mecillinam	Cefsulodin + Mecillinam		Cefsulodin + Mecillinam	
			5 : 1	10 : 1	5 : 1	10 : 1
S. aureus 308 A-1 (10^8)	3.46 (2.71~ 4.51)	174 (125 ~ 247)	3.75 (2.88 ~ 4.93)	4.65 (3.76 ~ 5.86)	0.91	1.22
E. coli O-111 (10^5)	39.1 (31.4 ~ 47.3)	0.044 (0.035~ 0.053)	0.227 (0.183~ 0.274)	0.330 (0.243~ 0.418)	0.87	0.69
E. coli T 7 (10^4)	268 (222 ~328)	9.62 (7.45 ~ 12.3)	41.4 (34.7 ~ 49.4)	70.5 (59.1 ~84.1)	0.85	0.91
C. freundii TN 518 (10^5)	20.7 (17.5 ~ 24.6)	0.401 (0.338~ 0.473)	1.32 (1.05 ~ 1.73)	2.13 (1.79 ~ 2.53)	0.60	0.56
K. pneumoniae DT (10^8)	32.4 (26.2 ~ 39.9)	6.18 (4.44 ~ 10.5)	7.59 (5.48 ~ 11.0)	7.26 (5.97 ~ 8.84)	0.40	0.31
E. cloacae TN 603 (10^4)	58.0 (47.6 ~ 70.7)	0.738 (0.539~ 0.936)	2.41 (1.96 ~ 2.95)	3.79 (3.06 ~ 4.73)	0.59	0.53
S. marcescens TN 66 (10^8)	118 (96.7 ~142)	>800	48.5 (39.3 ~ 59.5)	56.3 (46.0 ~69.0)	0.35	0.44
P. vulgaris GN 4712 (10^2)	186 (146 ~247)	54.3 (39.9 ~ 78.6)	75.1 (58.2 ~100)	73.5 (57.9 ~93.7)	0.57	0.48
P. morganii TN 373 (10^8)	54.1 (45.5 ~ 64.6)	649 (366 ~2,740)	32.9 (24.0 ~ 43.7)	30.4 (24.9 ~37.5)	0.51	0.51
P. aeruginosa U 31 (10^8)	5.43 (3.77~ 7.10)	>800	7.74 (5.40 ~ 10.4)	6.01 (4.14 ~ 7.95)	1.19	1.01

a) Mice were infected intraperitoneally with test organism 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.

in the peritoneal washing and blood of mice treated with 100 mg of cefsulodin per kg and 200 mg of mecillinam per kg was compared with that of each antibiotic alone (Fig. 3). In mice treated with cefsulodin or mecillinam alone, the increase in the number of CFU in the peritoneal washing and blood was greater than that in the control mice. By the combination of the two antibiotics, the number of CFU in the peritoneal washing decreased to about 10 CFU/ml at 1 hour after treatment and the same level was maintained for up to 8 hours. No bacterial count could be made in the blood of mice treated with the combination of cefsulodin and mecillinam.

Fixed-combination

The effect of the combination of cefsulodin and mecillinam in the ratio of 5: 1 and 10: 1 in mice infected intraperitoneally with 10 bacterial strains in 9 species is shown in Table 7. A synergistic effect (FED index; lower than 0.5) was observed in mice infected with *K. pneumoniae* DT and *S. marcescens* TN 66. In mice infected with other test bacterial strains, the FED index ranged from about 0.5 ~ 1.2 and these findings indicate that the combination of the two antibiotics resulted in an additive effect.

Discussion

The combination of cefsulodin with other antibiotics is of potential clinical interest because cefsulodin has a very narrow antibacterial spectrum. A potent antibacterial activity of cefsulodin has been observed on *P. aeruginosa* and *S. aureus* and the activity against other Gram-negative rods is weak. The mode of action of cefsulodin against *P. aeruginosa* is different from that of many β-lactam antibiotics against Gram-negative rods but that against *E. coli*, which showed weak susceptibility, was similar to that of β-lactam antibiotics[8]. Mecillinam has an antibacterial activity against certain Gram-negative rods[4~7] and shows a unique mode of action[9]. In the present study, the observed effects of the combination of these antibiotics, which have different antibacterial spectra and different sites of action on cell wall synthesis suggest synergistic action. This is supported by two different sets of data. First, the combination of cefsulodin and mecillinam produced an expansion of the antibacterial spectrum. This suggests that the combination of the two antibiotics does have a synergistic effect against several bacterial species. Second, the effect of the combination of the two antibiotics when examined by the checkerboard dilution method against selected bacterial strains, the fixed combination method against many clinical isolates, and the killing kinetics against certain bacterial strains also suggests a synergistic effect. In particular, a marked synergistic effect was observed on *S. marcescens* and *P. mirabilis*. The results in the fixed combination test were interesting, because the combination rates used in the test were established by the dose of each antibiotic used clinically and was not an optimal ratio resulting from the checkerboard dilution test. The killing kinetic test is the most prominent method for the analysis of the effect of combination but is an unsuitable method for studying effects against many strains.

The effect of a combination of mecillinam and broad spectrum β-lactam antibiotics has been reported on many members of *Enterobacteriaceae* and *Bacteroides* sp[10~27]. However, this effect is not universal and the mode of action involved is not clear. The antibacterial activity of β-lactam antibiotics against Gram-negative bacteria is thought to be the outcome of complex series of steps involving the permeability of the drug through the bacterial outer membrane, the stability to hydrolysis by β-lactamases and the inhibitory activity of the drug against the peptidoglycan synthesizing enzymes[28~33]. The mechanism of action of antibiotic combination has been classified into three types involving increased entry of the antibiotics into the organism in order to reach their sites of activity, preventing of the inactivation of one antibiotic by the other, and action by the antibiotics at two different points in a biochemical pathway[23]. Cefsulodin and mecillinam not only have a high resistance to hydrolysis by β-lactamases but a low affinity for enzymes[34,35]. These findings suggest that the interaction between the two antibiotics and β-lactamases is not involved in the promotion mechanism. The two antibiotics have different sites of activity in the cell wall synthesizing mechanisms. It is possible that two antibiotics affect two different points in the pathway of cell wall synthesis. These possibilities will be investigated.

A synergistic effect of cefsulodin plus mecillinam *in vitro* was observed on many bacterial species but in the *in vivo* test, a synergistic effect was observed on only two species, *S. marcescens* and *K. pneumoniae*. In mice infected with *S. marcescens* TN 66, the optimum ratio of the combination of the two antibiotics was different from that of the *in vitro* test. These results suggest that the effect of the combination must not only be tested *in vitro* but confirmed *in vivo*. SCHELD *et al.*[36] reported that the ampicillin-mecillinam combination was synergistic in a rabbit model of meningitis caused by *E. coli* but not synergistic in *K. pneumoniae* meningitis. More useful information may be presented by the test in model infections resembling clinical pictures. GRUNBERG and CLEELAND[5] reported that there were suitable antibiotic partners for combination with mecillinam. The findings presented here suggest that for development of a synergistic effect by two antibiotics, a suitable antibiotic combination is an important factor and a synergistic effect may be found against certain bacterial species but never against all bacterial strains.

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