IN VITRO EVALUATION OF SF-2103A, A NOVEL CARBAPENEM ANTIBIOTIC, AS A β-LACTAMASE INHIBITOR

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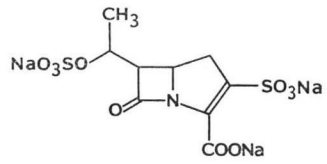
SF-2103A, a new carbapenem antibiotic, exhibited a broad antibacterial spectrum and a potent inhibitory activity against a wide range of β-lactamases, in particular, against cephalosporinases, with lower I_{50} values than those displayed by sulbactam and clavulanic acid. Using a fixed combination and checkerboard titration, *in vitro* synergy against the majority of the β-lactamase-producing strains tested was demonstrated between SF-2103A and various β-lactam antibiotics, especially cefotaxime, ceftizoxime, and cefoperazone. The synergistic effect of SF-2103A was more pronounced than that of sulbactam. The *in vitro* synergy was also confirmed by bactericidal and bacteriolytic activities and morphological effects.

The successful clinical application of combinations of clavulanic acid or sulbactam with β-lactam antibiotics has attracted attention for β-lactamase inhibitors as chemotherapeutic agent. SF-2103A is a novel carbapenem antibiotic produced by *Streptomyces sulfonofaciens* sp. nov., showing high β-lactamase-inhibitory activity, in particular against cephalosporinases produced by Gram-negative bacteria[1]. Its high stability in aqueous solution and its successful large scale preparation by fermentation enabled us to evaluate SF-2103A as a β-lactamase inhibitor, in conjunction with various β-lactam antibiotics. In this paper, extensive *in vitro* evaluation of SF-2103A combined with eight cephalosporins and one penicillin is described, using sulbactam and clavulanic acid as reference β-lactamase inhibitors.

Chart 1. Chemical structure of SF-2103A, trisodium 7-oxo-3-sulfo-6-[1-(sulfoxy)ethyl]-1-azabicyclo[3.2.0] hept-2-ene-2-carboxylate.



Materials and Methods

Drugs

Antibiotic SF-2103A was produced by *S. sulfonofaciens* SF-2103, and was purified according to the procedure already reported[1]. Sulbactam and cefminox (CMNX, MT-141) were synthesized, and clavulanic acid was prepared by fermentation of *Streptomyces* sp. SF-1932 in this laboratory. Cefotaxime was from Hoechst Japan Limited, Tokyo, cefoperazone from Toyama Chemical Co., Ltd., Tokyo, ceftizoxime and cefazolin from Fujisawa Pharmaceutical Co., Ltd., Osaka, cefmetazole from Sankyo Co., Ltd., Tokyo, and cephalothin and ampicillin from Meiji Seika Kaisha, Ltd., Tokyo.

Bacterial Strains

Organisms used were stock cultures of this laboratory, and kept at −20°C in 10% skim milk (Difco Laboratories, Detroit) before use. Many of the β-lactamase-producing strains have been

characterized by Professors YAMAGISHI and SAWAI, Chiba University[2]. The R plasmids RGN14 and RGN823 mediate RICHMOND type III (Ia or TEM-1 and Ib or TEM-2 penicillinases, respectively according to MITSUHASHI & SAWAI). The R plasmid RGN238 mediates type V (II or OXA-2) penicillinase.

Determination of MICs

Minimum inhibitory concentrations (MICs) were determined by the two-fold agar dilution method, using sensitivity-disc agar (modified Mueller-Hinton, Nissui Seiyaku, Tokyo), unless otherwise stated. The cultures were grown overnight in sensitivity test broth (Eiken Chemicals, Tokyo) at 37°C to give the inoculum cultures (10^8 cfu/ml). These were diluted 100-fold with buffered saline to give 10^6 cfu/ml.

One loopful (*ca.* 5 μl) of the culture was inoculated by means of a microplanter (Sakuma Seisakusho, Tokyo) onto 15 ml of agar containing serial two-fold dilutions of the test antibiotics or the combinations. The agar plates were incubated at 37°C for 20 hours. The MIC was defined as the lowest drug concentration that inhibited development of visible growth on agar. A slight haze or the presence of up to three colonies was ignored.

Synergy in the fixed combination was defined by a four-fold or greater reduction in MICs of both drugs, and partial synergy by a four-fold or greater reduction of MIC of one drug and a two-fold reduction in MIC of the other drug. The fractional inhibitory concentration (FIC) index in the checkerboard titration method[3] was calculated according to ELION *et al.*[4]. A sign of inequality was ignored for the calculation of FIC index. Synergy was defined by FIC index <0.5, and additivity by $2 \geqq \text{FIC} \geqq 0.5$, antagonism by $\text{FIC} > 2$.

Preparation of Crude β-Lactamase

An overnight culture of a test strain (4 ml) was inoculated into 2% bouillon broth (Kyokuto Seiyaku, Tokyo), and incubated at 32°C for 6 hours. For inducible β-lactamases, benzylpenicillin at 250 μg/ml was added at the start of incubation and 2 and 4 hours later. The cells were harvested in the logarithmic phase of growth, and centrifuged at $10{,}000 \times g$ for 5 minutes at 4°C. The cells thus obtained were washed twice with 0.1 M phosphate buffer (pH 7.0), resuspended in a two-fold volume of the buffer and disrupted at 4°C with an ultrasonic disintegrater (Waken Co., Ltd., Tokyo). The cell debris was removed by centrifugation at $12{,}000 \times g$ for 10 minutes. The supernatant was dialyzed overnight against 0.1 M phosphate buffer at 4°C, and stored at −20°C before use.

Measurement of β-Lactamase-inhibitory Activity

The inhibitory activity against hydrolysis by β-lactamase was determined by a modified microiodometric method[5]. An inhibitor solution (0.05 ml) in 0.1 M phosphate buffer (pH 7.0) was mixed with a substrate solution (0.05 ml) containing 2 mM benzylpenicillin in a case of a penicillinase or 2 mM cephalothin in a case of a cephalosporinase. The mixture was kept at 30°C for 5 minutes, added to a β-lactamase solution (0.9 ml) in 0.1 M phosphate buffer, and allowed to react at 30°C for 30 minutes. The reaction was stopped by the addition of 0.5 ml of 0.15 M sodium tungstate in 2 M acetate buffer (pH 4.0). The iodine-starch reagent (1.5 ml) consisting of 0.1 mM iodine, 1.6 mM potassium iodide, 0.4% starch and 0.04 M phosphate buffer (pH 6.0) was added. After standing for 10 minutes at room temperature, absorbance at 490 nm was measured.

In case of preincubation, an inhibitor solution (0.05 ml) was reacted with a β-lactamase solution (0.9 ml) at 30°C for 10 minutes before mixing with a substrate solution (2 mM, 0.05 ml). The degree of β-lactamase inhibition was expressed as 50% inhibition concentration (I_{50}) of a control which was measured without inhibitor.

Viable Cell Count

L-Tubes containing 10 ml of heart infusion broth (Difco) and 10^{-3} dilutions of the preculture were incubated at 37°C for 1 hour with shaking, and a test cephalosporin and/or inhibitor were added. The mixed cultures were incubated at 37°C for 8 hours with gentle shaking. Viable cells were counted at 1, 2, 4, 6 and 24 hours after incubation, and 0.1-ml quantities of ten-fold serial dilutions of the culture fluid were spread on heart infusion agar plates. The colonies were counted after incubation at

Table 1. Antibacterial spectra of SF-2103A, sulbactam and clavulanic acid.
Medium was sensitivity-disc agar with inoculum of 10^6 cfu/ml.

Organism	MIC (μg/ml)		
	SF-2103A	Sulbactam	Clavulanic acid
Staphylococcus aureus 209P JC-1	6.25	50	3.13
S. aureus Smith	6.25	100	12.5
S. epidermidis ATCC 14990	6.25	>100	6.25
Enterococcus faecalis ATCC 8043	6.25	>100	>100
Bacillus subtilis ATCC 6633	1.56	50	25
Escherichia coli ML1410	3.13	50	25
E. coli NIHJ JC-2	0.78	25	25
Klebsiella pneumoniae GN69*	3.13	25	25
K. pneumoniae PCI 602	3.13	25	25
Salmonella typhi O-901-W	3.13	50	50
S. enteritidis No. 11	0.78	50	50
Shigella dysenteriae Shigae	1.56	25	25
Proteus vulgaris OX-19	6.25	>100	100
Providencia rettgeri J-0026*	12.5	>100	100
Enterobacter cloacae G-0006*	25	>100	50
Serratia marcescens No. 2	3.13	100	100
Pseudomonas aeruginosa MB-3833	100	>100	100
P. aeruginosa E-2	50	>100	100
Xanthomonas maltophilia M-0627	>100	>100	50

* β-Lactamase producers.

37°C for 18 hours.

The synergy in the killing kinetics was defined by the decline of viable cells 100 times or more from that occupied with either agent alone[6].

Measurement of Cell Density

An automatic biophotometer, MS-2 Research System (Abbott Laboratories, Irving, Texas) was used with plastic cuvettes of 1 ml capacity to measure growth and lysis of the cells. Heart infusion broth (0.9 ml) inoculated with 50 μl of an overnight cultures of test strains was pre-incubated at 37°C for 5 hours. An aqueous solution of test antibiotics or their combinations was added, and the cuvettes were incubated at 37°C for 5 hours to record the time course of optical density at 660 nm. The synergy in the bacteriolysis was arbitrarily defined by the reduction of optical density at least 0.2 from that occurred with either agent alone.

Phase-contrast Microscopy

One percent of the overnight culture of *Proteus vulgaris* GN76/C-1 was inoculated into sensitivity test broth (20 ml). The mixture was incubated at 37°C for 3 hours on a rotatory shaker at 60 rpm. A suspension of the shaken cultures in the logarithmic growth phase was smeared over a cover glass. This was combined with a glass slide on which a 3-mm thick sensitivity disc agar (Nissui) containing a drug or the combination of two drugs was layered. This was embedded in paraffin and incubated at 37°C. Observation and photography were performed with a reverse type phase-contrast microscope (Nikon).

Results

Antibacterial and β-Lactamase-inhibitory Activity

Table 1 shows the antibacterial spectrum of SF-2103A, sulbactam and clavulanic acid. SF-2103A exhibited a broad antibacterial spectrum with moderate activity against Gram-positive and Gram-negative bacteria. The susceptible strains included β-lactamase-producing organisms, indicat-

Table 2. β-Lactamase-inhibitory activity of SF-2103A, sulbactam and clavulanic acid.

Source of β-lactamase	Type of β-lactamase[a]	Preincubation time (minutes)	I_{50} (μg/ml)		
			SF-2103A	Sulbactam	Clavulanic acid
Escherichia coli 255	Ib (CSase)[b]	0	0.048	>50	>10
		10	0.0035	8.4	>10
Proteus vulgaris GN76/C-1	Ic (CSase)	0	0.0036	1.1	0.35
		10	0.00015	0.21	0.0026
Citrobacter freundii GN346	Ia (CSase)	0	0.0086	>50	>10
		10	0.00068	3.9	>10
Enterobacter cloacae GN7471	Ia (CSase)	0	0.0088	>50	>10
		10	0.0015	8.9	>10
Serratia marcescens GN10857	Ia (CSase)	0	0.13	25	>10
		10	0.020	1.4	>10
Pseudomonas aeruginosa GN10362	Ia (CSase)	0	0.24	26	>10
		10	0.047	3.3	>10
Staphylococcus aureus MS258	(PCase)[c]	0	>50	29	2.8
		10	4.0	1.1	
E. coli W3630 RGN823	IIIa (Ib) (PCase)	0	1.6	1.3	0.13
		10	0.05	0.68	0.0089
E. coli W3630 RGN238	V (II) (PCase)	0	13	>50	>10
		10	0.0013	0.88	0.29
P. aeruginosa M-0148	(PCase)[d]	0	1.3	2.1	0.14
		10	0.053	0.78	0.0084

[a] RICHMOND classification. Classification according to MITSUHASHI and SAWAI was shown in parenthesis.
[b] Abbreviation of cephalosporinase.
[c] Abbreviation of penicillinase.
[d] Carbenicillinase.

ing that SF-2103A was stable to the β-lactamases. The antibacterial activity of SF-2103A was superior to those of the reference inhibitors, except in case of Staphylococci where it was equal to that of clavulanic acid.

Table 2 shows the β-lactamase-inhibitory activity of SF-2103A, sulbactam and clavulanic acid. As judged from I_{50} values, the inhibitory activity of SF-2103A was far more potent than the reference inhibitors against type I cephalosporinases, and was comparable to the references against various other types of penicillinases except for staphylococcal penicillinase. In all the cases, the inhibition was markedly increased after preincubation, thereby showing that SF-2103A behaves as a progressive inhibitor.

Synergistic Antibacterial Activity of Combinations of SF-2103A or Sulbactam with β-Lactam Antibiotics

Synergy in 1: 2 Fixed Combinations of Inhibitors with Four Cephalosporins and One Penicillin

Table 3 shows antibacterial activity of the 1: 2 fixed combinations of SF-2103A or sulbactam with cefotaxime, cefoperazone, cefminox, cephalothin and ampicillin. The MICs were determined with heavy inocula to amplify the effect of β-lactamase inhibition. Among 20 strains tested, twelve were cephalosporinase, and the remaining eight penicillinase producers. With the cephalosporins SF-2103A appeared more synergistic against cephalosporinase than against penicillinase producers.

This was most typical for the combinations with cefotaxime, cefminox and cephalothin, where synergy was observed only against the cephalosporinase producers. Cefoperazone showed the highest frequency of synergy, including cephalosporinase- and penicillinase-producers. Ampicillin showed synergy with SF-2103A against both types of β-lactamase producers. Among the Gram-negative strains tested, *P. vulgaris*, *Morganella morganii*, *Citrobacter freundii* and *Escherichia coli* yielded synergy more often than *Enterobacter cloacae* and *Serratia* species.

Sulbactam as the reference inhibitor showed comparable synergy with the test antibiotics, but the number of strains that showed synergy was less than that with SF-2103A.

The 1: 1 combinations of SF-2103A or sulbactam with the five β-lactams mentioned above showed a synergistic effect very close to that shown by the 1: 2 combination (data not shown).

FIC Indices of Combinations of SF-2103A or Sulbactam with Five Cephalosporins and One Penicillin

Table 4 shows a list of FIC indices of the combinations between SF-2103A or sulbactam with cefotaxime, cefoperazone, ceftizoxime, cefmetazole, cefazolin and ampicillin. As judged from the FIC indices against 15 strains, most of the β-lactam antibiotics were synergistic with SF-2103A, showing FIC indices of $0.003 \sim 0.5$. Among the six β-lactam antibiotics, cefoperazone showed the highest synergy with SF-2103A, and cefmetazole the lowest. Cefotaxime, ceftizoxime, cefazolin and ampicillin showed intermediate degrees of synergy.

Among the test strains, the synergistic effect of SF-2103A was best demonstrated against *P. vulgaris* GN76/C-1, except for the combination with cefmetazole, and then against *E. coli* GN206, *E. cloacae* GN7471 and *M. morganii* 1510. On the other hand, the synergistic activity with cephalosporins against penicillinase producers was weak except for cefoperazone and cefazolin. Ampicillin showed equal synergy against both types of β-lactamases producers.

The synergistic effect of sulbactam was similar to but unfrequent than that of SF-2103A. A much greater amount of sulbactam than that of SF-2103A was required to reduce the MICs of the cephalosporins to the same level.

Table 3. Antibacterial activity of the 1: 2 combinations of SF-2103A or sulbactam with cefotaxime (CTX), cefoperazone (CPZ), cefminox (CMNX), cephalothin (CET) and ampicillin (ABPC).
Medium: Heart infusion agar (Eiken); inoculum 10^8 cfu/ml.

Organism	Type of β-lactamase	MIC (μg/ml)							
		SF-2103A	Sulbactam	CTX	SF-2103A + CTX	Sulbactam + CTX	CPZ	SF-2103A + CPZ	Sulbactam + CPZ
Escherichia coli 255	CSase (Ib)	12.5	50	100	6.25^{+}	50	800	3.13^{++}	25^{+}
E. coli GN206	CSase (Ib)	12.5	50	6.25	1.56^{++}	3.13	25	0.39^{++}	1.56^{++}
Proteus vulgaris GN76/C-1	CSase (Ic)	50	>100	100	0.20^{++}	0.39^{++}	800	1.56^{++}	3.13^{++}
P. vulgaris GN106	CSase	50	>100	0.20	0.20	0.10^{+}	6.25	0.78^{++}	3.13^{+}
Morganella morganii 1510	CSase	50	>100	100	12.5^{++}	12.5^{++}	800	12.5^{++}	25^{++}
M. morganii Kono	CSase	100	>100	25	25	12.5^{+}	100	25^{++}	100
Citrobacter freundii GN346	CSase (Ia)	100	100	50	50	50	400	50^{+}	100
C. freundii GN346/16-10	CSase (Ia)	12.5	100	12.5	6.25	12.5	200	3.13^{++}	12.5^{++}
Enterobacter cloacae G-0006	CSase	>100	>100	12.5	100	25	>1,600	50^{++}	25^{++}
E. cloacae G-0005	CSase	25	>100	12.5	6.25	12.5	>1,600	12.5^{+}	25^{++}
Serratia sp. GN629	CSase	12.5	>100	>100	>100	100	>1,600	25	>100
S. marcescens No. 1	CSase	25	>100	>100	25	100	>1,600	50	>100
Staphylococcus aureus 606	PCase	50	>100	3.13	3.13	1.56^{+}	12.5	12.5	6.25^{+}
E. coli W3630 RGN823	PCase (IIIa)	6.25	100	0.20	0.10^{+}	0.20	>1,600	3.13^{+}	50^{+}
E. coli W3630 RGN14	PCase	6.25	50	0.20	0.10^{+}	0.20	200	0.78^{++}	6.25^{++}
E. coli W3630 RGN238	PCase (V)	12.5	50	0.20	0.20	0.20	0.20	0.39	0.39
Klebsiella pneumoniae GN69	PCase (IIIa)	12.5	100	0.20	0.20	0.20	>1,600	1.56^{++}	25^{++}
K. pneumoniae GN118	PCase	100	>100	3.13	12.5	12.5	3.13	1.56^{+}	12.5
Proteus mirabilis GN79	PCase	>100	>100	50	50	50	>1,600	50	50
Pseudomonas aeruginosa M-0148	PCase	>100	>100	100	>100	25^{++}	>1,600	>100	>100
Total number of synergistic strains					3	3		10	8

Table 3. (Continued)

Organism	Type of β-lactamase	MIC (μg/ml)							
		CMNX	SF-2103A + CMNX	Sulbactam + CMNX	CET	SF-2103A + CET	ABPC	SF-2103A + ABPC	Sulbactam + ABPC
Escherichia coli 255	CSase (Ib)	100	6.25^{+}	50	3,200	12.5	800	25^{++}	>100
E. coli GN206	CSase (Ib)	50	6.25^{+}	25	3,200	6.25^{+}	400	6.25^{++}	50
Proteus vulgaris GN76/C-1	CSase (Ic)	0.78	1.56	1.56	>3,200	12.5^{++}	>3,200	>100	>100
P. vulgaris GN106	CSase	1.56	1.56	1.56	>3,200	3.13^{++}	3,200	50^{+}	100^{++}
Morganella morganii 1510	CSase	25	6.25^{++}	12.5^{+}	>3,200	100	1,600	50^{+}	>100
M. morganii Kono	CSase	25	6.25^{++}	12.5^{+}	>3,200	100	400	100^{+}	50^{+}
Citrobacter freundii GN346	CSase (Ia)	400	50^{+}	100	>3,200	50^{+}	1,600	25^{++}	>100
C. freundii GN346/16-10	CSase (Ia)	25	3.13^{++}	25	1,600	12.5	200	50^{+}	100
Enterobacter cloacae G-0006	CSase	800	100^{+}	>100	>3,200	>100	>3,200	>100	>100
E. cloacae G-0005	CSase	800	25	>100	>3,200	50	>3,200	100^{+}	>100
Serratia sp. GN629	CSase	200	50	50^{++}	>3,200	>100	>3,200	>100	>100
S. marcescens No. 1	CSase	12.5	6.25^{+}	12.5	>3,200	50	100	50	100
Staphylococcus aureus 606	PCase	25	25	50	0.39	0.78	3,200	25	12.5^{++}
E. coli W3630 RGN823	PCase (IIIa)	0.78	0.78	0.78	200	6.25	>3,200	25^{+}	>100
E. coli W3630 RGN14	PCase	0.78	0.78	0.78	25	6.25	>3,200	50^{+}	50^{++}
E. coli W3630 RGN238	PCase (V)	0.78	0.78	1.56	12.5	6.25	25	25	25
Klebsiella pneumoniae GN69	PCase (IIIa)	0.78	1.56	1.56	25	12.5	>3,200	>100	>100
K. pneumoniae GN118	PCase	1.56	3.13	3.13	25	50	1,600	12.5^{++}	25^{++}
Proteus mirabilis GN79	PCase	12.5	25	25	50	>100	>3,200	>100	>100
Pseudomonas aeruginosa M-0148	PCase	800	100	100	>3,200	>100	>3,200	>100	>100
Total number of synergistic strains			3	1		2		4	4

$^{++}$; Synergistic strains, $^{+}$; partially synergistic strains.

Table 4. Synergistic activity of SF-2103A and sulbactam in combination with cefotaxime (CTX), cefoperazone (CPZ), ceftizoxime (CZX), cefmetazole (CMZ), cefazolin (CEZ) and ampicillin (ABPC).

Organism	MIC (μg/ml)							
	SF-2103A	Sulbactam	CTX	CPZ	CZX	CMZ	CEZ	ABPC
Escherichia coli 255	3.13	50	25	100	100	50	>1,600	400
E. coli GN206	3.13	50	12.5	50	25	50	1,600	200
Proteus vulgaris GN76/C-1	12.5	>100	>400	>1,600	12.5	6.25	>1,600	>1,600
Morganella morganii 1510	25	>100	50	400	200	50	>1,600	800
Citrobacter freundii GN346	12.5	50	50	1,600	200	50	>1,600	800
Enterobacter cloacae GN7471	6.25	50	50	200	400	800	>1,600	400
Serratia marcescens GN10857	50	>100	200	>1,600	>400	400	>1,600	>1,600
Bacteroides fragilis No. 5	12.5	25	25					
Staphylococcus aureus 606	12.5	100	1.56	12.5	0.78	1.56	6.25	1,600
S. aureus MS258	12.5	100	3.13	6.25	3.13	1.56	1.56	400
E. coli W3630 RGN823	1.56	100	0.20	>1,600	0.10	1.56	800	>1,600
E. coli No. 29/36 RGN823	3.13	100	0.05	>1,600	0.05	1.56	400	>1,600
E. coli W3630 RGN238	3.13	25	6.25	12.5	12.5	3.13	12.5	1,600
Klebsiella pneumoniae GN69	3.13	100	0.20	1,600	0.05	1.56	25	>1,600
Pseudomonas aeruginosa M-0148	>100	>100	25	>1,600	50	>1,600	>1,600	>1,600

Organism	FIC index			
	SF-2103A+CTX	Sulbactam+CTX	SF-2103A+CPZ	Sulbactam+CPZ
Escherichia coli 255	0.13 (0.39: 3.13)*	1.0 (50: 0.20)	0.06 (0.10: 6.25)	0.50 (12.5: 50)
E. coli GN206	0.25 (0.78: 0.05)	0.28 (12.5: 0.39)	0.02 (0.025: 0.78)	0.07 (3.13: 0.78)
Proteus vulgaris GN76/C-1	0.07 (0.78: 0.05)	0.03 (3.13: 0.78)	0.01 (0.025: 1.56)	0.27 (25: 25)
Morganella morganii 1510	0.26 (6.25: 0.39)	0.25 (25: 0.20)	0.08 (1.56: 6.25)	0.09 (6.25: 12.5)
Citrobacter freundii GN346	0.50 (3.13: 12.5)	0.63 (6.25: 25)	0.13 (1.56: 12.5)	0.31 (12.5: 100)
Enterobacter cloacae GN7471	0.38 (0.78: 12.5)	0.50 (12.5: 12.5)	0.08 (0.10: 12.5)	0.19 (25: 12.5)
Serratia marcescens GN10857	0.31 (12.5: 12.5)	0.63 (50: 25)	0.19 (3.13: 200)	0.50 (25: 400)
Bacteroides fragilis No. 5	0.09 (0.39: 1.56)	0.13 (0.10: 3.13)		
Staphylococcus aureus 606	0.50 (3.13: 0.39)	0.53 (3.13: 0.78)	0.38 (3.13: 1.56)	0.14 (1.56: 1.56)
S. aureus MS258	1.02 (12.5: 0.05)	0.50 (25: 0.78)	0.37 (1.56: 1.56)	0.27 (1.56: 1.56)
E. coli W3630 RGN823	0.63 (0.78: 0.025)	0.53 (3.13: 0.10)	0.16 (0.20: 50)	0.08 (6.25: 25)
E. coli No. 29/36 RGN823	0.63 (0.39: 0.025)	0.63 (12.5: 0.025)	0.07 (0.20: 12.5)	0.07 (6.25: 50)
E. coli W3630 RGN238	0.13 (0.39: 0.05)	0.16 (3.13: 0.20)	0.06 (0.10: 0.39)	0.19 (1.56: 1.56)
Klebsiella pneumoniae GN69	0.50 (0.78: 0.05)	0.50 (25: 0.05)	0.04 (0.10: 6.25)	0.13 (12.5: 12.5)
Pseudomonas aeruginosa M-0148			0.50 (25: 400)	
No. of strains showing FIC <0.5	8/14	5/14	13/14	11/13

Table 4. (Continued)

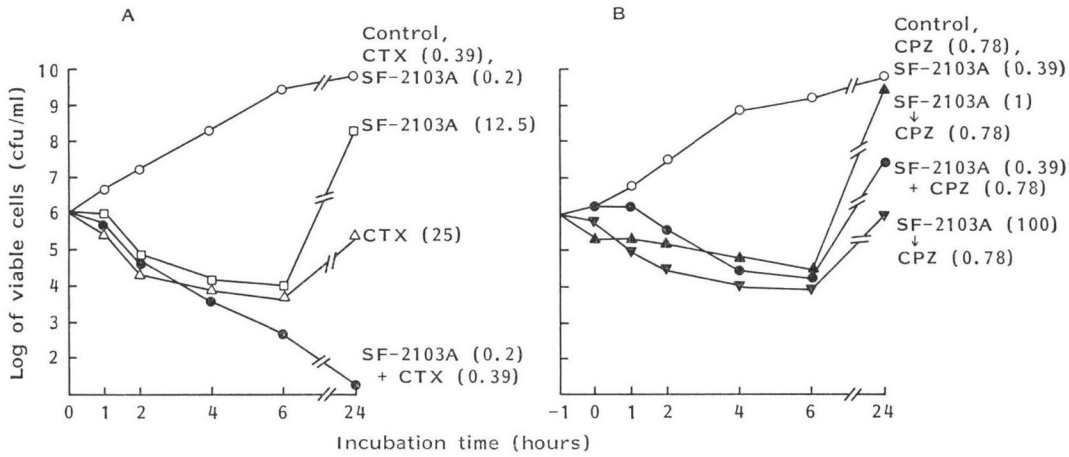
Organism	FIC index			
	SF-2103A+CZX	Sulbactam+CZX	SF-2103A+CMZ	Sulbactam+CMZ
Escherichia coli 255	0.26 (0.78: 1.56)	0.52 (25: 1.56)	0.50 (0.78: 12.5)	0.75 (12.5: 25)
E. coli GN206	0.16 (0.39: 0.78)	0.50 (25: 0.025)	0.52 (0.39: 3.13)	0.38 (6.25: 12.5)
Proteus vulgaris GN76/C-1	0.003 (0.025: 0.012)	0.01 (0.78: 0.05)	0.51 (0.78: 3.13)	1.06 (100: 0.39)
Morganella morganii 1510	0.28 (6.25: 6.25)	0.38 (25: 25)	0.16 (0.78: 6.25)	0.19 (6.25: 6.25)
Citrobacter freundii GN346	0.28 (3.13: 6.25)	0.50 (12.5: 50)	0.19 (1.56: 1.56)	1.25 (12.5: 50)
Enterobacter cloacae GN7471	0.13 (0.39: 25)	0.38 (12.5: 50)	0.16 (1.56: 25)	0.38 (12.5: 100)
Serratia marcescens GN10857	0.25 (12.5: 1.56)	0.28 (25: 12.5)	0.56 (3.13: 200)	
Bacteroides fragilis No. 5				
Staphylococcus aureus 606	0.51 (3.13: 0.20)	0.75 (50: 0.20)	0.63 (1.56: 0.78)	0.75 (25: 0.78)
S. aureus MS258	0.38 (3.13: 0.39)	0.37 (25: 0.39)	0.63 (1.56: 0.78)	0.50 (25: 0.39)
E. coli W3630 RGN823	0.38 (0.20: 0.025)	0.56 (6.25: 0.05)	2.00 (1.56: 1.56)	1.00 (50: 0.78)
E. coli No. 29/36 RGN823	0.32 (0.39: 0.012)	1.12 (100: 0.006)	0.75 (1.56: 0.39)	1.13 (100: 0.20)
E. coli W3630 RGN238	0.05 (0.10: 0.20)	0.12 (1.56: 0.78)	0.56 (0.20: 1.56)	0.75 (12.5: 0.78)
Klebsiella pneumoniae GN69	0.32 (0.39: 0.10)	0.63 (12.5: 0.025)	0.63 (1.56: 0.20)	0.63 (50: 0.20)
Pseudomonas aeruginosa M-0148	1.0 (0.39: 50)			
No. of strains showing FIC <0.5	12/14	6/13	3/13	3/12

Organism	FIC index		
	SF-2103A+CEZ	Sulbactam+CEZ	SF-2103A+ABPC
Escherichia coli 255	0.14 (0.39: 25)	0.31 (12.5: 100)	0.50 (0.78: 100)
E. coli GN206	0.13 (0.39: 12.5)	0.13 (3.13: 100)	0.31 (0.78: 12.5)
Proteus vulgaris GN76/C-1	0.02 (0.20: 12.5)	0.19 (12.5: 100)	0.01 (0.20: 3.13)
Morganella morganii 1510	0.09 (1.56: 50)	0.25 (12.5: 200)	0.16 (0.78: 100)
Citrobacter freundii GN346	0.62 (1.56: 800)	0.56 (25: 100)	0.62 (1.56: 400)
Enterobacter cloacae GN7471	0.25 (0.78: 200)	0.56 (25: 100)	0.37 (1.56: 50)
Serratia marcescens GN10857	2.00 (50: 1,600)		0.63 (25: 200)
Bacteroides fragilis No. 5			
Staphylococcus aureus 606	0.19 (0.78: 0.78)	0.08 (1.56: 0.39)	0.38 (3.13: 200)
S. aureus MS258	0.37 (1.56: 0.39)	0.28 (0.39: 0.39)	0.28 (3.13: 12.5)
E. coli W3630 RGN823	0.14 (0.20: 12.5)	0.05 (3.13: 12.5)	
E. coli No. 29/36 RGN823	0.10 (0.20: 12.5)	0.14 (1.56: 50)	1.0 (3.13: 800)
E. coli W3630 RGN238	0.19 (0.20: 1.56)	0.13 (3.13: 3.13)	0.31 (0.20: 400)
Klebsiella pneumoniae GN69	0.13 (0.20: 1.56)	0.16 (3.13: 3.13)	0.53 (1.56: 50)
Pseudomonas aeruginosa M-0148	2.0 (100: >1,600)		
No. of strains showing FIC <0.5	11/14	10/12	7/12

* Numbers in parenthesis indicate MICs (μg/ml) of inhibitor and β-lactam in combination, from which minimal FIC index was calculated.

Fig. 1. Time courses of bactericidal effect of the combination of SF-2103A with cefotaxime (CTX) (A) or cefoperazone (CPZ) (B) against *Proteus vulgaris* GN76/C-1.

Figures in parenthesis indicate drug concentration (μg/ml), and arrow indicates pretreatment with SF-2103A for 10 minutes followed by treatment with cefoperazone alone.



Synergy of Bactericidal and Bacteriolytic Activities and Morphological Effects

In order to examine the mode of the synergistic action, the bactericidal and bacteriolytic activities and morphological effects of the combinations were studied *in vitro*. Fig. 1 shows the bactericidal effect of the 1: 2 fixed combinations of SF-2103A with cefotaxime (A) or cefoperazone (B) at the MIC level against *P. vulgaris* GN76/C-1 which produces type Ic cephalosporinase. Apparently, the combination of SF-2103A with cefotaxime at the concentration of 0.39 μg/ml showed synergistic bactericidal effect. When compared with cefotaxime alone or SF-2103A alone, the combination caused a maximal decrease of 10^8 cfu/ml of the viable cells after 24 hours incubation. The combination was more bactericidal than any of the single components concentrated 62.5 times.

The combination of SF-2103A with cefoperazone also showed synergistic bactericidal effect at the concentration of 0.78 μg/ml. But, the degree of killing was moderate compared with that given by the cefotaxime combination. The maximal difference between the combination and either component alone was 10^4 cfu/ml.

Interestingly, similar bactericidal activity was observed after short pretreatment with SF-2103A. Treatment of the culture with 1 or 100 μg/ml of SF-2103A for 10 minutes followed by collecting the cells by centrifugation and addition of fresh cefoperazone resulted in suppression of bacterial growth at least for 6 hours. This indicated that the effect of pretreatment with SF-2103A lasted at least for 6 hours after the removal of SF-2103A.

Fig. 2 shows the bacteriolytic effect of the 1: 2 combinations of SF-2103A with cefotaxime or cefoperazone. The bacteriolytic activity of both combinations was synergistic, showing reduction of optical density of 0.25~0.35. In parallel with the bactericidal activity, cefotaxime showed a more marked lytic effect than cefoperazone, when combined with SF-2103A. This indicates that the killing activity of the combination is affected by the nature of cephalosporin partner.

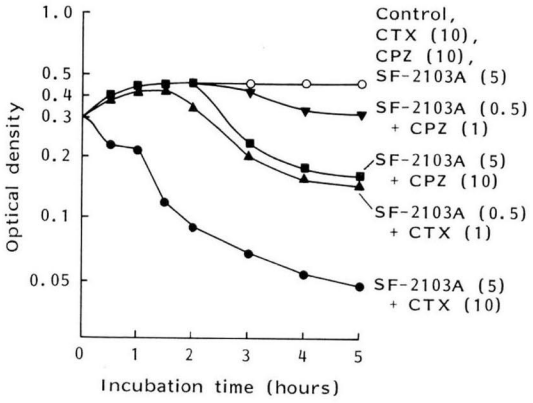
Fig. 3 shows the bactericidal (A) and bacteriolytic (B) effects of the 1: 2 combination of SF-2103A

with cefoperazone against *E. coli* GN206 which produces type Ib cephalosporinase. The synergistic effect of the combination was evident after 6 hours exposure in the bactericidal effect, and at 4~5 hours in the lytic effect. The effect of pretreatment with SF-2103A for 10 minutes was seen even at 24 hours.

Fig. 4 shows the morphological changes of *P. vulgaris* GN76/C-1 cells exposed to the 1:2 combinations of SF-2103A with cefotaxime or cefoperazone. In the case of the combination of SF-2103A with cefotaxime, the cells were elongated, causing bulges around the middle part of the cells after 3 hours treatment at 0.39 μg/ml (MIC) and 1.56 μg/ml (4×MIC), whereas little change was observed after treatment of either agent alone at similar concentrations. At higher concentration (400 μg/ml) shown in the figure, cefotaxime alone caused bulges at the middle part of the filamentous cells, similar to those seen with the combination, and SF-2013A alone at 12.5 μg/ml caused bulges at the termini of the filamentous cells. SF-2103A was shown to be bound preferably to PBP-1A, 3 and 4 of *E. coli* K-12, but a direct relation of the PBP binding and the morphological change was not obvious (T. TSURUOKA, private communication).

Fig. 2. Time courses of bacteriolytic effect of the combination of SF-2103A with cefotaxime (CTX) or cefoperazone (CPZ) against *Proteus vulgaris* GN76/C-1.

Figures in parenthesis indicate drug concentration (μg/ml).



The combination of SF-2103A with cefoperazone caused the elongation of the cells and subsequent formation of bulges at 0.78 μg/ml (MIC) and 3.13 μg/ml (4×MIC), similar to cefoperazone alone at 400 μg/ml.

These results suggested that the cephalosporins played a main role in determining the antibacterial activity of the 1:2 combination, and that SF-2103A acted as a β-lactamase inhibitor, rather than as an

Fig. 3. Time courses of bactericidal effect (A) and bacteriolytic effect (B) of the combination of SF-2103A with cefoperazone (CPZ) against *Escherichia coli* GN206.

Figures in parenthesis indicate drug concentration (μg/ml), and arrow indicates pretreatment with SF-2103A.

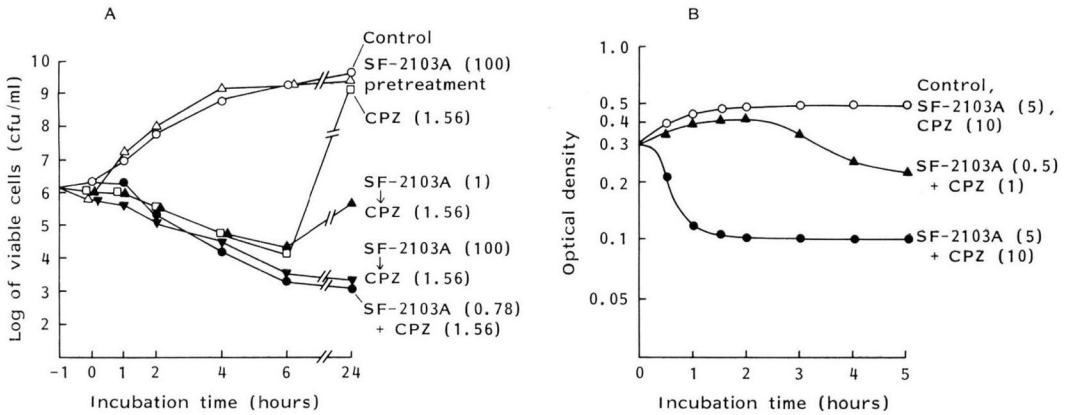
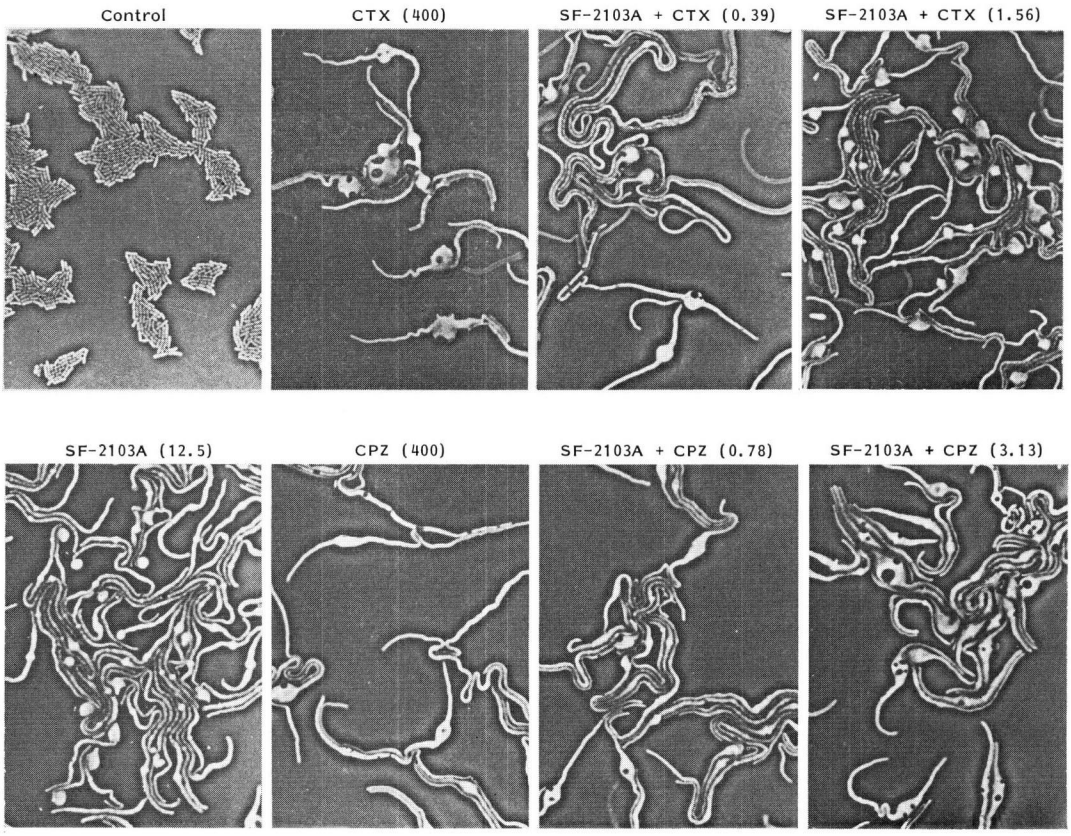


Fig. 4. Micrographs of *Proteus vulgaris* GN76/C-1 cells exposed to the combination of SF-2103A with cefotaxime (CTX) or cefoperazone (CPZ) at 37°C for 3 hours.
Figures in parenthesis indicate drug concentration (μg/ml).



antibacterial agent.

Discussion

More than 30 carbapenem antibiotics have been reported, many of which show potent β-lactamase-inhibitory activity[7,8]. However, reports on the synergism of carbapenems combined with β-lactam antibiotics are rather a few. Only preliminary reports on the combinations of olivanic acids with amoxicillin[9], PS-5 with ampicillin and cephaloridine[10], asparenomycin A with ampicillin[11] and C-19393 with ampicillin and cefotiam[12] have been published. Accordingly, this paper appears to be the first extensive study on the synergism of a carbapenem antibiotic as a β-lactamase inhibitor in combination with β-lactam antibiotics, in particular cephalosporins.

It is evident from Tables 2, 3 and 4 that SF-2103A is an active inhibitor of chromosomally mediated cephalosporinases, more active than sulbactam and clavulanic acid. In addition, it appeared as active as the latter two inhibitors against the limited range of penicillinases tested except the staphylococcal enzyme.

However, the synergistic effect of SF-2103A in conjunction with cephalosporins was not as large as expected from the I_{50} values. This was true especially against *C. freundii*, *E. cloacae* and *Serratia marcescens* in the 1 : 2 fixed combination. As YAMAGUCHI *et al.* suggested[13], the poor synergy against *C. freundii* may be due to rapidly reversible inhibition. On the other hand, the synergy was high against *P. vulgaris*, where the blocking of the β-lactamase is long enough to allow the β-lactam antibiotic to reach its target.

The poor synergy with cefmetazole and cefminox against *P. vulgaris* may be due to the fact that the two cephamycins themselves are quite stable to the *P. vulgaris* β-lactamase. Similarly, the poor synergy of the cephalosporin combinations against penicillinase producers may be ascribed to the high stability of test cephalosporins. However, it is of interest that cefoperazone and cefazolin as well as ampicillin showed good synergy against penicillinase producers. The poor synergy against *Pseudomonas aeruginosa* may be due to poor penetration of SF-2103A to the cells, since SF-2103A was quite inhibitory to the cell-free penicillinases of *Pseudomonas* origin. The results suggest that cefotaxime, ceftizoxime and especially cefoperazone are good partners of SF-2103A for synergistic activity.

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