ASPARENOMYCINS A, B AND C, NEW CARBAPENEM ANTIBIOTICS

IV. ANTIBACTERIAL ACTIVITY

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Asparenomycins (ASM) A, B and C, new members of the carbapenem family of antibiotics, are broad spectrum antibiotics with activity against Gram-positive and Gram-negative bacteria. ASM A was bactericidal to both aerobic and anaerobic bacteria, although morphological alterations of ASM A exposed cells differed significantly between *Escherichia coli* and *Bacteroides fragilis*; with the former ovoidal forms were produced while with the latter elongated forms were seen. Synergistic activities were observed with a combination of ASM A and ampicillin (ABPC) against various ABPC-resistant bacteria presumably as a result of the inhibition by ASM A of β-lactamases.

ASM A showed relatively weak therapeutic activity against *E. coli* infected mice, because of instability in body fluids, a common property of the carbapenem family of antibiotics.

Asparenomycins (ASM) A, B and C were isolated from the culture broths of *Streptomyces tokunonensis* and *Streptomyces argenteolus*[1,2]. Structural assignment[3] revealed that the ASMs belong to the carbapenem family which includes thienamycin[4], epithienamycin[5], PS-5[6], the carpetimycins[7] and the olivanic acid group.[8] This paper describes the *in vitro* and *in vivo* antibacterial activities of ASM A, B and C.

Materials and Methods

Antibiotics

Asparenomycins (ASM) A, B and C were prepared by Shionogi Research Laboratories.[2] Ampicillin sodium (ABPC) was the product of Meiji Seika Co.

Organisms

Bacterial cultures were laboratory strains maintained in our laboratories and recent clinical isolates identified in our laboratories.

In Vitro Antibacterial Activity

The minimum inhibitory concentration (MIC) was determined by the agar dilution method as previously reported[9]. The medium for aerobic bacteria was Sensitivity Test (ST) agar (Eiken Co.). For streptococci, ST agar containing horse serum (5%) was used. The anaerobic bacteria were tested on GAM agar[9] and incubated in an anaerobic chamber (N_2, 80%; H_2, 10%; CO_2, 10%). Bacterial cultures containing approximately 10^6 viable cells/ml were prepared from overnight cultures. One loopful of the culture was inoculated on the agar plates containing antibiotics. The agar plates were incubated at 37°C for 18 to 24 hours and the MIC value was determined.

Microbiological Assay

Concentrations of ASM were determined by the agar-well assay method, using *Escherichia coli* 7347 as test organism. Agar plates containing organisms (10^5 viable cells/ml) were each prepared by pouring 10 ml of melted nutrient agar into 9 cm Petri dishes. Wells were bored in each solidified agar

by removing agar plugs of 6-mm diameter. A 20-μl aliquot of the sample or of the standard solution was added to the well. After incubation at 37°C for 20 hours, the diameters of inhibition zones were measured and the amounts of antibiotic in the test samples were calculated from the standard curves.

Bactericidal Action

A twofold dilution series of the antibiotic was prepared in heart infusion broth, using a microtiter plate (96 wells; Cooke Engineering, U. S. A.). A bacterial suspension at the exponential growth phase was prepared by subculturing diluted overnight cultures for 2 hours. An 50-μl aliquot of the suspension was inoculated into each well. Final populations of inoculum were adjusted to approximately 10^6 viable cells/ml. After various intervals, samples of the cultures were taken and transplanted to the surface of nutrient agar by delivering 0.5 μl. The 99% killing concentration (99% KC) was defined as the lowest concentration of antibiotic permitting growth of less than five colonies on subculture after incubation for 24 hours. Thus, the killing rate at the minimal bactericidal concentration was at least 99%. The original plates of the broth culture were incubated for 24 hours, and the MICs were determined. All incubations were performed at 37°C.

The overnight broth cultures of *B. fragilis* were diluted in GAM broth and incubated at 37°C. When the cultures reached the exponential growth phase, approximately 10^7 viable cells/ml, they were divided into 1-ml samples, and twofold dilutions (9 ml) of ASM A in GAM broth were added to give the desired final concentrations. Incubation was continued at 37°C in the anaerobic chamber. Samples of cultures with or without antibiotic were removed at appropriate intervals and viability was determined by agar pouring method.

Morphological Response of Antibiotic-exposed Bacteria

The overnight culture of *E. coli* was diluted 100-fold in trypto-soy broth and incubated for 1~2 hours at 37°C. One loopful of the bacterial suspension (10^7 viable cells/ml) was inoculated on heart infusion agar containing doubling dilutions of an antibiotic. After exposure of the culture for various intervals at 37°C, microscopic observations were made by phase-contrast microscopy, and morphological changes in exposed cells were recorded. For *B. fragilis*, the bacterial suspension was prepared from the overnight culture in GAM broth using the anaerobic chamber. Anaerobic conditions were kept while exposing the organisms to the antibiotic.

Animals

Female ICR strain mice aged 5 weeks and weighing 18~20 g were used for both pharmacological studies and protection tests.

Determination of Plasma Levels

Mice were given 20 mg/kg of the antibiotic by subcutaneous injection. Four mice were sacrificed at each interval. Blood samples were taken by cardiac puncture and plasma were separated by centrifugation for bioassay of the antibiotic.

Stability in Body Fluids

The blood plasma, kidney and liver were removed from the mice. A 10% homogenate of each organ was prepared in 0.1 M phosphate buffer solution (pH 7.0). After 0.01 ml of the antibiotic solution was mixed with a 1 ml portion of the homogenate or plasma to make the initial concentration at 10 μg/ml. Every 30 minutes during incubation at 37°C, aliquots were added to an equal volume of ethanol and the antibiotic activity assayed.

Protection Test

Acute lethal infections in mice were produced by intraperitoneal injection of *E. coli* EC-14 suspended in 5% gastric mucin (ICN Pharmaceuticals, Inc.). The number of organisms to induce uniform lethality in nontreated mice within 24 hours was predetermined as previously reported[9]. The test antibiotic was dissolved in distilled water and administered subcutaneously at 1 and 5 hours after infection. Five to ten mice were used for each dose level. The 50% effective dose (ED_{50}) was calculated by probit analysis from the survival rate on the 7th day.

Table 1. Minimum inhibitory concentrations (μg/ml) against aerobic bacteria.

Organism	ASM A	ASM B	ASM C	ABPC
Staphylococcus aureus 209P JC-1	1.56	6.25	6.25	0.1
S. aureus C-14*	1.56	12.5	6.25	6.25
Streptococcus pyogenes C-203	1.56	0.78	0.78	0.05
Escherichia coli NIHJ JC-2	1.56	3.13	0.78	6.25
E. coli 377**	0.39	3.13	0.39	100
E. coli 73*	3.13	12.5	0.78	>100
Klebsiella pneumoniae SRL-1	0.78	6.25	0.78	0.78
Proteus mirabilis PR-4	3.13	6.25	1.56	1.56
P. morganii 9	25	50	6.25	50
P. vulgaris CN-329	12.5	12.5	1.56	50
Enterobacter cloacae 233	1.56	12.5	1.56	50
Serratia marcescens ATCC 13880	12.5	50	3.13	25
Pseudomonas aeruginosa ATCC 25619	25	>100	12.5	>100

* Penicillinase producing strain; ** cephalosporinase producing strain.

Results and Discussion

Antibacterial Spectrum

Table 1 compares the *in vitro* antibacterial activities of the three components of asparenomycin and of ampicillin against aerobic bacteria. The asparenomycins were active against a wide range of both Gram-positive and Gram-negative bacteria. ASM A and ASM C exhibited similar spectra and were superior to ASM B in antibacterial activity against various organisms. ASM C tended to be more active against Gram-negative bacteria including β-lactamase-producing organisms than ASM A. Antipseudomonal activity was observed in two components, but only against strains that were highly sensitive to carbenicillin. ASM A was tested for activity against obligate anaerobic bacteria (Table 2): most strains were inhibited by concentrations greater than 6.25 μg/ml. As ASM A was very stable to β-lactamase produced by *B. fragilis*, the MICs against the strains were equivalent despite large disparities in amounts of β-lactamase produced.

Table 2. Minimum inhibitory concentrations (μg/ml) against obligate anaerobic bacteria.

Organism	ASM A	ABPC
Peptococcus anaerobius ATCC 14956	25	0.2
Peptostreptococcus anaerobius B-38	6.25	0.78
Eubacterium limosum ATCC 8486	100	0.1
Clostridium perfringens ATCC 13123	0.39	0.025
Bacteroides fragilis R-1-20	25	12.5
B. fragilis V-176*	25	100
B. thetaiotaomicron IMA 9	6.25	25
B. distasonis Ju-11-1	6.25	6.25
B. vulgatus WAL 1887	12.5	12.5
B ovatus 2505	12.5	12.5
Fusobacterium varium ATCC 8501	50	3.13
F. nucleatum FN-1	6.25	0.05
Veillonella parvula ATCC 10790	6.25	0.05

* Cephalosporinase producing strain.

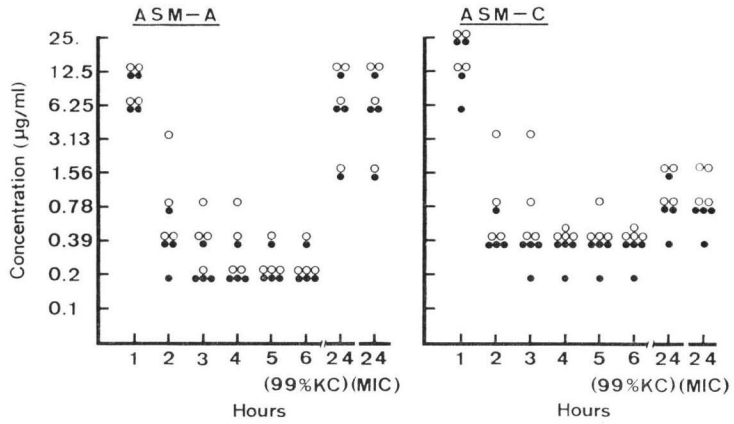
Bactericidal Activity

Time course experiments on the 99% killing concentrations of ASM A and ASM C were performed with 8 strains of *E. coli* including four ABPC-resistant strains and four ABPC-sensitive strains (Fig. 1). In Fig. 1 each plot represents the concentration of antibiotic required to give 99% kill after exposure for the given interval for each isolate. Both components were highly bactericidal against the strains regardless of whether they produced β-lactamase or not. The short-term killing effects of ASM A and ASM C were

Fig. 1. Time course of 99% killing concentration (99% KC) of asparenomycin A (left) and C (right) against 8 strains of *E. coli* clinically isolated.

Each point represents the minimum concentration required to kill one strain at least 99% killing rate.

Symbols: ●, ampicillin-susceptible strains; ○, ampicillin-resistant strains.

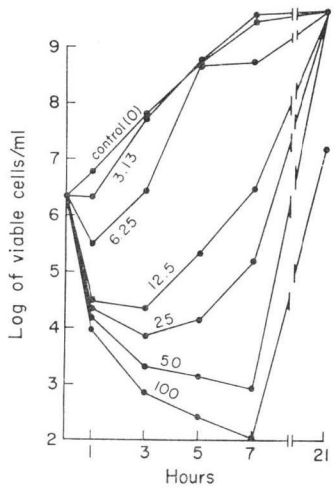


produced at 0.2 and 0.39 μg/ml, respectively. Whereas, after 24 hours, ASM A was consistently less bactericidal than ASM C, with the 99% killing concentrations ranging from 1.56 to 12.5 μg/ml for ASM A and from 0.78 to 1.56 μg/ml for ASM C. This discrepancy of the bactericidal kinetics is probably explained by the stability of both antibiotics in broth culture.

Bactericidal activity of ASM A against *B. fragilis* R-1-20 is shown in Fig. 2. Reduction of viability was quite rapid. After 1 hour of incubation, viability decreased by 100-fold at concentrations over 12.5 μg/ml. Subsequent onset of growth was delayed with period of delay dependent on the concentration of ASM A employed. As the new cells were sensitive to ASM A, the regrowth appeared to be due to the instability of ASM A in the culture.

Fig. 2. Bactericidal activity of asparenomycin A against *B. fragilis* R-1-20 tested in GAM broth.

Numbers indicate concentrations in μg/ml.



Morphological Alterations of Organisms

E. coli NIHJ JC-2 and *B. fragilis* R-1-20 were exposed to twofold dilutions of ASM A and the morphological changes observed in phase-contrast microscopy (Figs. 3 and 4, respectively). When *E. coli* cells were incubated for 2 hours with ASM A at a concentration of 0.2 μg/ml, round and ovoid forms appeared with no change in population numbers (Fig. 3-b). At 0.78 μg/ml, cells had a swollen appearance and decreased in population numbers. Most of the cells were lysed without an alteration of morphology at 6.25 μg/ml. As subinhibitory concentrations of mecillinam are known to induce ovoidal

Fig. 3. *E. coli* NIHJ JC-2 after 2 hours of untreated control cells and exposed cells with ASM A. (a) Untreated control; (b) ASM A, 0.2 μg/ml; (c) ASM A, 0.78 μg/ml; (d) ASM A, 6.25 μg/ml (1×MIC). Bar, 10 μ.

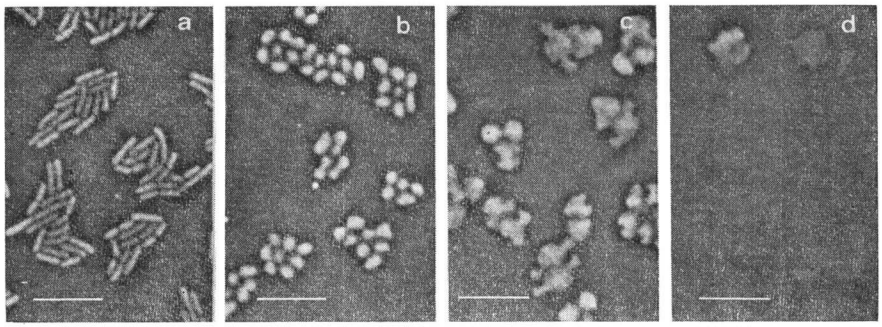
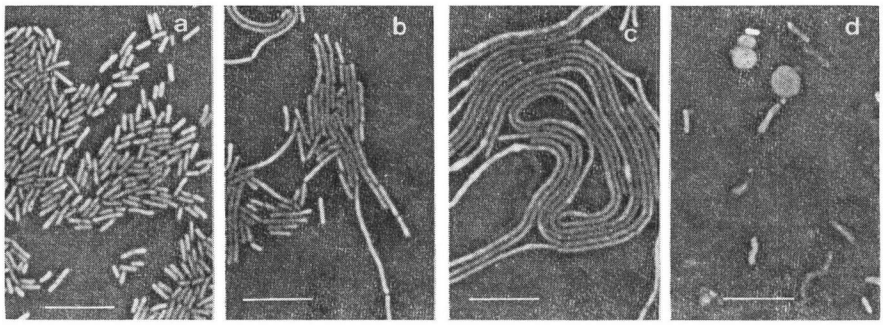


Fig. 4. *B. fragilis* R-1-20 after 5 hours of untreated control cells and exposed cells with ASM A. (a) Untreated control; (b) ASM A, 0.39 μg/ml; (c) ASM A, 1.56 μg/ml; (d) ASM A, 6.25 μg/ml (1/4×MIC). Bar, 10 μ.



forms and no filaments in *E. coli*[10], the mode of action of ASM A on *E. coli* may be similar to that of mecillinam. In contrast, ASM A treatment of *B. fragilis* produced filaments but no round cells at subinhibitory concentrations ranging from 0.39 to 3.13 μg/ml (Fig. 4-b and 4-c). Lysis of cells was seen after formation of spheroplast-like forms by exposure to 1/4 of the MIC of ASM A for 5 hours (Fig. 4-d). These morphological responses were initiated even after one hour exposure to ASM A. However, ovoid forms were not observed at any time interval. The morphological alterations in *B. fragilis* resembled the elongated forms of Gram-negative bacilli induced by most penicillins[11]. Since the effects of β-lactam antibiotics on the morphology of *E. coli* are known to be related to their binding affinities for three different bacterial proteins[12] and if the same penicillin binding proteins are present in both *B. fragilis* and *E. coli*, one could speculate the binding affinity of ASM A to these proteins differ between the two species.

Synergism of Asparenomycin A with Ampicillin

Synergism of antibacterial activity by ASM A combination with ABPC was studied using ABPC-resistant bacteria in the checker board titration technique, since ASM A was noted to be a potent inhibitor of β-lactamases[13] (Table 3). The synergistic effect was estimated by the minimum FIC index as previously reported[14]. The combinations were considered to be synergistic when there was a 4-fold or greater reduction in the MIC values of two antibiotics in comparison to the individual activity. Against

Table 3. Synergistic effect of asparenomycin A on antibacterial activity of ampicillin.

Organism	Strain	MIC (μg/ml)*				Synergism** coefficient
		Alone		Combination		
		ASM A	ABPC	ASM A + ABPC		
S. aureus	3131	>10	100	2.5	12.5	1
	3132	>10	100	2.5	12.5	2
P. vulgaris	31	>10	>200	1.25	25	2
	50	>10	>200	0.63	12.5	3
E. cloacae	44	>10	>200	1.25	100	1
	50	10	>200	1.25	100	1
S. marcescens	39	10	>200	2.5	3.1	2
	45	10	>200	2.5	100	1
B. fragilis	R-1-20	3.13	50	<0.1	1.56	3
	V-261-1	6.25	800	0.39	<12.5	3

* Broth dilution method was employed for *B. fragilis* and agar dilution method for other bacteria. Incubation time: 5 hours for *B. fragilis* and 20 hours for other bacteria.

** Synergism coefficient was defined from minimum FIC index[14] indicated in parentheses: 1=(0.5 ~ >0.25), 2=(0.25 ~ >0.1), 3=(≦0.1).

the organisms listed in Table 3, the combinations of ASM A and ABPC were found to be synergistic. As the organisms employed were highly resistant to ABPC and produced β-lactamases, synergism appeared to be due to inhibition of β-lactamases and was particularly pronounced in cephalosporinase-producing organisms. In contrast *E. coli* and *Klebsiella* sp. resistant to ABPC because of their production of penicillinase type β-lactamase were found to be sensitive equally to ASM A with or without ABPC.

In Vivo Antibacterial Activity

The therapeutic efficacy of ASM A was studied against an intraperitoneal infection of *E. coli* EC-14 in mice. The ED_{50} value was determined to be 11.5 mg/kg/dose a value higher than that predicted from the MIC (0.78 μg/ml) for the strain, when compared to results with other β-lactam antibiotics[4].

The plasma level profile in mice was determined after subcutaneous injection at a dose of 20 mg/kg. At 15 and 30 minutes after dosing, the concentrations in plasma reached 1.5 and 0.35 μg/ml, respectively. As the peak level at the effective dose was approximately equal to the MIC value, the relatively low plasma concentrations could be responsible to its weak therapeutic efficacy.

Stability of Asparenomycin A in Body Fluids

The results of stability tests of ASM A in mouse plasma and tissue homogenates at 37°C are presented in Table 4. Decrease in activity was very rapid in the body fluids tested and particularly so in the kidney homogenate. It has been reported that a kidney enzyme efficiently hydrolyzes thienamycin-type compounds[5]; a similar mechanism may be involved in the case of ASM A. The low urinary recovery of ASM A, less than 0.5% of administered dose, may be the result of inactivation in the kidney.

Table 4. Stability of asparenomycin A at 37°C*.

Mice	Half life in minutes
Plasma (99%)	50
Liver homogenates (10%)	90
Kidney homogenates (10%)	15

* Initial concentration: 10 μg/ml.

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