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Studies on Novel Bacterial Translocase I Inhibitors, A-500359s

II. Biological Activities of A-500359 A, C, D and G

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A-500359 A, C, D, G and capuramycin inhibited bacterial phospho-*N*-acetylmuramyl-pentapeptide-translocase (translocase I: EC 2.7.8.13) with IC_{50} values of 0.017, 0.12, 0.53, 0.14 and 0.018 μM, respectively. Consistently, A-500359 A, C and capuramycin inhibited *in vitro* peptidoglycan biosynthesis. A-500359 A exhibited reversible inhibition, which was mixed type and noncompetitive with respect to UDP-MurNAc-(N^{ε}-Dns)pentapeptide (Ki=0.0079 μM) and undecaprenyl-phosphate, respectively. A-500359 A, C, D and G showed antimicrobial activity against *Mycobacterium smegmatis*. As a single intravenous injection of A-500359 A at a dose of 500 mg/kg showed no toxicity in mice, it was suggested that the capuramycin derivatives might become candidates as novel therapeutic agents for various diseases caused by Mycobacteria including tuberculosis.

In the preceding paper, we described the taxonomy of the producing strain, SANK 60196, and the fermentation, isolation, physico-chemical properties and structure elucidation of A-500359 A, C, D and G. In this paper, we report the biological activities of A-500359 A, C, D, G and capuramycin.

Materials and Methods

Materials

Undecaprenyl-phosphate was purchased from Larodan Fine Chemicals. *E. coli* JM109 harboring pTA5, a *mraY* expression vector, was a generous gift from Dr. MASATO IKEDA[1]. Luria Broth (LB) and Trypto-Soy Broth (TSB; Eiken Chemical Co., LTD.) were used for the cultivation of the bacteria. UDP-*N*-acetyl-[*U*-^{14}C]glucosamine (UDP-[^{14}C]GlcNAc; 200 mCi/mmol) was purchased from Amersham.

Fluorescence was measured with Fluoroskan Ascent (Labsystems), a fluorescence spectrophotometer, at room temperature.

Preparation of Translocase I

E. coli JM109/pTA5 was grown in LB media containing 50 μg/ml of ampicillin at 37°C to the growth stage of $OD_{600\,nm}$ 2.9. The cells harvested by centrifugation were washed with 50 mM Tris-HCl buffer (pH 8.0) containing 0.1 mM $MgCl_2$, and re-suspended in the same buffer at 4°C. After disruption of the cells by sonication, the remaining cells and debris were removed by centrifugation (5,000×g, 10 minutes) and membrane fragments were collected by ultracentrifugation (105,000×g, 60 minutes) at 4°C. The pellet was washed with the same buffer and re-suspended in solubilization buffer consisting of 50 mM Tris-HCl (pH 8.0), 0.1 mM $MgCl_2$, 1% Triton X-100 and 30% (v/v) glycerol. The suspension was stirred for 30 minutes at 4°C to solubilize the enzyme. After removal of insoluble materials by ultracentrifugation using the same conditions as described above, the resulting crude enzyme solution was divided into 0.5 ml portions, and stored at −80°C until use.

Preparation of Fluorescent Substrate

UDP-*N*-acetylmuramyl-L-Ala-γ-D-Glu-*m*-DAP-D-Ala-D-

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Ala (UDP-MurNAc-pentapeptide) was isolated from *Bacillus cereus* SANK 70880 cells based on the methodology devised by LUGTENBERG *et al.*[2] and FLOURET *et al.*[3]. UDP-MurNAc-L-Ala-γ-D-Glu-*m*-DAP-(N^{ε}-dansyl)-D-Ala-D-Ala (UDP-MurNAc-[N^{ε}-Dns]pentapeptide) was prepared by treatment of the natural substrate with dansyl chloride (WAKO) in 50% aqueous acetone essentially as described by WEPPNER and NEUHAUS[4].

Measurement of Translocase I Inhibitory Activity

The method described by BRANDISH *et al.*[5] was partially modified as follows. Assay constituents were added to each well in a 96-well microtitre polystyrene plate (Corning Coaster, #3694) in the following order. Fifty microliters of 200 mM Tris-HCl buffer (pH 7.5) containing 100 mM KCl and 50 mM $MgCl_2$ were added to each well followed by addition of 12 μl of water and 10 μl of 3.15 mg/ml UDP-MurNAc-(N^{ε}-Dns)pentapeptide. Eight microliters of lipid solution containing 2.5 mg/ml phosphatidylglycerol, 3.65 mg/ml undecaprenyl-phosphate and 10% (v/v) Triton X-100 were added to the assay mixture followed by addition of the test sample. The reaction was started by addition of 20 μl of the stored enzyme solution (0.625~2.5 μg protein). The increase in fluorescence concomitant with the formation of lipid-linked product was monitored at 538 nm (excitation at 355 nm).

Assay of Bacterial Peptidoglycan Synthesis *In Vitro*

E. coli NIHJ JC-2 cells at the late log phase of growth in TSB was harvested by centrifugation (5,000×*g*, 10 minutes) and treated with diethyl ether to make partially digested cells by the procedure of VOSBERG and HOFFMANN-BERLING[6]. A reaction mixture containing 100 μl of 50 mM Tris-HCl (pH 8.3), 50 mM NH_4Cl, 20 mM $MgCl_2$, 1 mM dithiothreitol, 10 mM ATP, 5.25 μM UDP-[^{14}C]GlcNAc (14 μCi/μmol) and 100 μM UDP-MurNAc-pentapeptide was added to each 1.5-ml tube followed by addition of the test sample. The reaction was started by addition of ether-treated cells corresponding to approximately 0.25 mg of protein. Tubes were incubated for 30 minutes. Precipitates were collected onto a Whatman MM paper disk, and the disk was washed 3 times with iced 5% trichloroacetic acid, ethanol and diethyl ether, successively. Radioactivity on the disk was counted with Picofluor (Packard).

Antimicrobial Activities

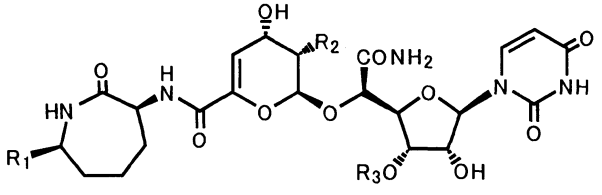
MICs were determined by the agar dilution method using Nutrient agar (Eiken Chemical Co., LTD.)

Results

Inhibition of Translocase and Peptidoglycan Synthesis by A-500359 A, C, D, G and Capuramycin

A-500359 A, C, D, G and capuramycin (Fig. 1) inhibited translocase I with IC_{50} values of 0.017, 0.12, 0.53, 0.14 and 0.018 μM, respectively (Table 1). Since translocase I plays an essential role in bacterial peptidoglycan biosynthesis, inhibitory activity of A-500359 A, C and capuramycin against *in vitro* peptidoglycan biosynthesis was examined. As shown in Table 1, these compounds inhibited incorporation of radio-labeled UDP-GlcNAc into cell wall fractions with IC_{50} values of 0.68, 6.1 and 0.53 μM, respectively.

Fig. 1. Structures of A-500359 A, C, D, G and capuramycin.



	R_1	R_2	R_3
A-500359 A	CH_3	OH	CH_3
A-500359 B (Capuramycin)	H	OH	CH_3
A-500359 C	CH_3	OH	H
A-500359 D	CH3	H	CH_3
A-500359 G	H	OH	H

Table 1. Inhibition of translocase I and *in vitro* peptidoglycan biosynthesis by A-500359 A, C, D, G and capuramycin.

	IC_{50} (μM)	
	Translocase I	PGS[a]
A-500359 A	0.017	0.68
A-500359 C	0.12	6.1
A-500359 D	0.53	NT[b]
A-500359 G	0.14	NT
Capuramycin	0.018	0.53

[a]*In vitro* peptidoglycan synthesis.
[b]Not tested.

Table 2. Reversible inhibition of translocase I by A-500359 A.

Concentration (μM) of A-500359 A during		Enzyme activity
preincubation	enzyme assay	(% of control)
0	0	100
0.028	0.007	82.8
0	0.007	86.6
0.085	0.021	58.7
0	0.021	61.6
0.25	0.063	28.8
0	0.063	27.1
0.76	0.19	11.7
0	0.19	9.1
2.3	0.57	5.9
0	0.57	3.5

The enzyme was pre-incubated for 10 minutes at room temperature in Tris-HCl buffer (pH 8.0) with or without A-500359 A and the remaining activity was determined under the concentrations of A-500359 A adjusted as indicated.

Reversible Inhibition of A-500359 A against Translocase I

Since mureidomycin A[7,8] is known as a slow binding inhibitor of translocase I[5], the effect of pre-incubation with A-500359 A against enzyme activity was investigated. A-500359 A and the enzyme were pre-mixed for 10 minutes before the enzyme assay. As shown in Table 2, there was no evidence that pre-incubation increased the inhibitory activity of A-500359 A. Thus, A-500359 A exhibited reversible inhibition.

Kinetic Analysis of Translocase I Inhibition by A-500359 A

The mode of action of translocase I inhibition by A-500359 A was examined. We verified the concentration of UDP-MurNAc-(N^{ε}-Dns)pentapeptide and undecaprenyl-phosphate, as well as the concentrations of A-500359 A and tunicamycin[9] in assay mixtures. Lineweaver-Burk plots are given in Fig. 2. A-500359 A showed mixed type inhibition and noncompetitive inhibition with respect to UDP-MurNAc-(N^{ε}-Dns)pentapeptide (Fig. 2A) and undecaprenyl-phosphate (Fig. 2B), respectively. Tunicamycin also showed mixed type inhibition with respect to UDP-MurNAc-(N^{ε}-Dns)pentapeptide (Fig. 2C), whereas it was competitive with respect to undecaprenyl-phosphate (Fig. 2D). With respect to UDP-MurNAc-(N^{ε}-Dns)pentapeptide, *Ki* values of A-500359 A and tunicamycin were calculated to be 0.0079 μM and 3.1 μM, respectively.

Antimicrobial Activity of A-500359 A

Antimicrobial activities of A-500359 A, C, D, G and capuramycin are given in Table 3. All of these compounds showed antimicrobial activity against *Mycobacterium smegmatis*. Moreover, A-500359 A and capuramycin were active against *Streptococcus pneumoniae* and *S. pyogenes*, but inactive against other microorganisms tested so far. A-500359 A had more potent antimicrobial activity than capuramycin.

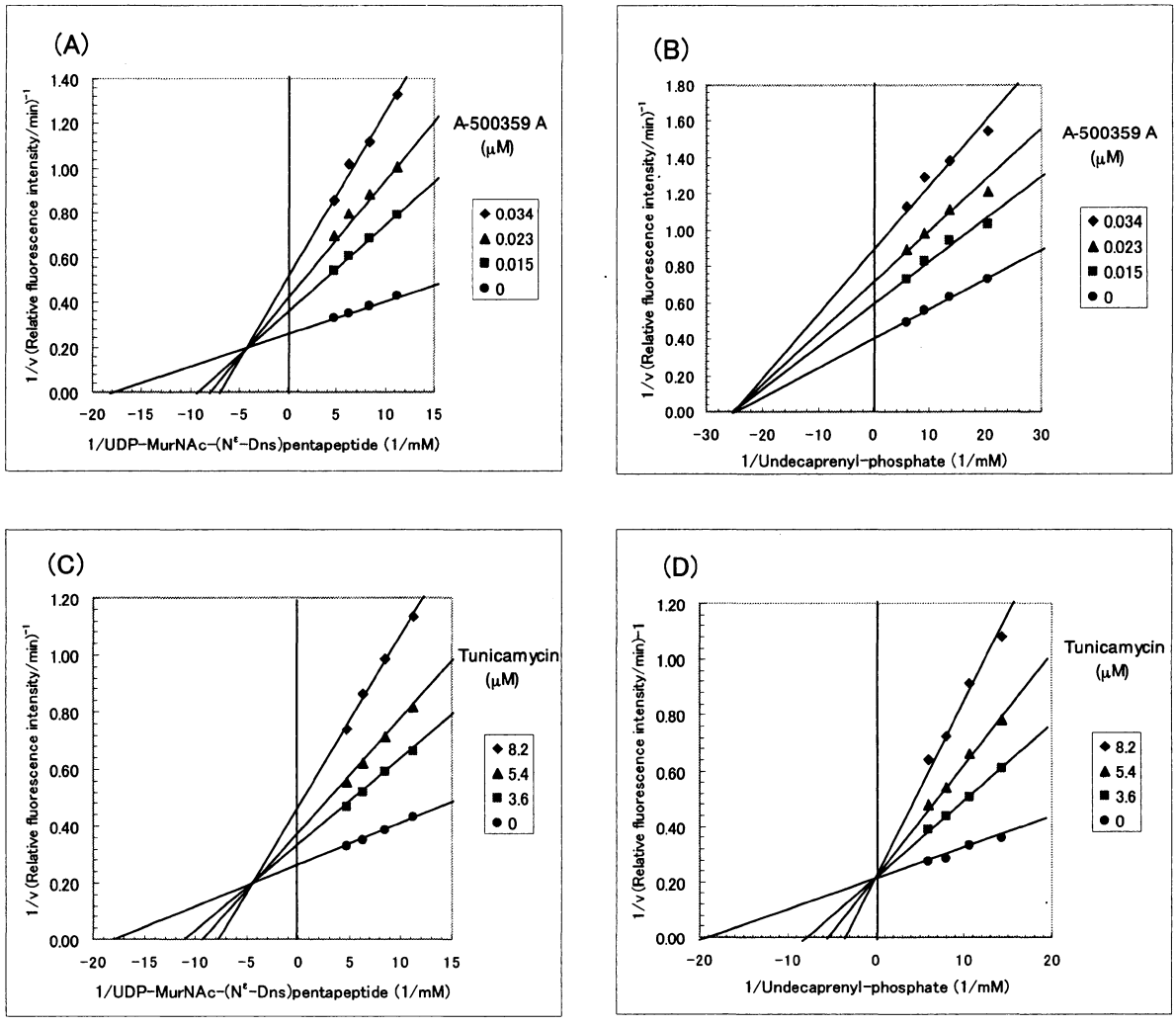
Toxicity

Mice tolerated a single intravenous injection of A-500359 A at 500 mg/kg without any toxic symptoms for 14 days after the treatment.

Discussion

Capuramycin was previously reported[10] as a nucleoside antibiotic produced by *Streptomyces griseus* in 1986, however, its molecular target had not been clarified. Even though various kinds of nucleoside antibiotics have been reported, this is the first report that translocase I is the molecular target of capuramycin in bacteria, and that its

Fig. 2. Kinetic analysis of translocase I inhibition by A-500359 A and tunicamycin.



Concentrations of UDP-MurNAc-(N^{ε}-Dns)pentapeptide (A, C) and undecaprenyl-phosphate NH_3 salt (B, D) were varied as indicated. The final enzyme concentration was 0.625 μg protein/ml.

analogues, A-500359 A, C, D and G also inhibit the enzyme activity. Among the capuramycin derivatives, A-500359 A and capuramycin were the most potent inhibitors with IC_{50} values of 0.017 and 0.018 μM, respectively. In addition, A-500359 A, C and capuramycin inhibited *in vitro* peptidoglycan biosynthesis consistently.

A-500359 A, C, D, G and capuramycin inhibited the growth of *M. smegmatis*. The antimicrobial activity of A-500359 A was the most potent compared with that of the others. Moreover, A-500359 A and capuramycin showed antimicrobial activity against *S. pneumoniae* and *S. pyogenes*. Among them, A-500359 A also showed the strongest antimicrobial activity against *S. pyogenes*. It was considered that the potency of the inhibition of translocase I by capuramycin derivatives basically correlated with their antimicrobial activity. Peptidyl-nucleoside type translocase inhibitors such as mureidomycins, pacidamycins and napsamycins show specific anti-*Pseudomonas* activity, whereas, capuramycin derivatives exhibited different antimicrobial spectra. The difference in the antimicrobial spectra was considered to be caused by the difference in permeability of these antibiotics or the compound selectivity of the efflux pump on each bacterium.

With respect to undecaprenyl-phosphate, A-500359 A

Table 3. Antimicrobial activity of A-500359 A, C, D, G and capuramycin.

Microorganisms	MIC (μg/ml)				
	A-500359 A	C	D	G	Capuramycin
Staphylococcus aureus FDA 209P JC-1	>100	>100	>100	>100	>100
Streptococcus pneumoniae SANK 73201[a]	12.5	-[c]	-	-	12.5
Streptococcus pneumoniae SANK 73301[b]	25	-	-	-	25
Streptococcus pyogenes SANK 73401	6.25	-	-	-	25
Bacillus subtilis ATCC 6633	>100	>100	>100	>100	>100
Mycobacterium smegmatis ATCC 607	6.25	25	100	50	12.5
Escherichia coli NIHJ JC-2	>100	>100	>100	>100	>100
Shigella flexneri IID642	>100	>100	>100	>100	>100
Klebsiella pneumoniae IID865	>100	>100	>100	>100	>100
Enterobacter cloacae IID977	>100	>100	>100	>100	>100
Serratia marcescens IAM1184	>100	>100	>100	>100	>100
Proteus vulgaris SANK 72890	>100	>100	>100	>100	>100
Pseudomonas aeruginosa SANK 73090	>100	>100	>100	>100	>100

[a]Penicillin sensitive strain.
[b]Penicillin high resistant strain.
[c]Not tested.

and tunicamycin were a noncompetitive inhibitor and a competitive inhibitor, respectively. Thus, the mode of action of translocase inhibition by A-500359 A was different from that by tunicamycin. The reason might be that tunicamycin has an alkyl side chain in its molecule while A-500359s does not. BRANDISH *et al.*[11] demonstrated a different result from that of ours, that tunicamycin showed noncompetitive inhibition with respect to undecaprenyl-phosphate. Further investigation is needed to clarify in more detail the mode of action of translocase I inhibitors.

Tunicamycin is known to inhibit not only translocase I but also mammalian enzymes that catalyze the formation of lipid-linked saccharides from nucleotide sugars and dolichyl-phosphate[9,12]. On the other hand, A-500359 A had no inhibitory activity to such mammalian enzymes up to 100 μg/ml (data not shown). These data indicate that A-500359 A is a specific inhibitor of translocase I.

A-500359 A is a specific inhibitor of translocase I, and is active against *Mycobacterium* sp. with no toxicity. Therefore, it is suggested that the capuramycin derivatives might become candidates for a novel therapeutic agent of various diseases caused by Mycobacteria including tuberculosis.

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