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Selective Inhibition of the Bacterial Peptidoglycan Biosynthesis by the New Types of Liposidomycins

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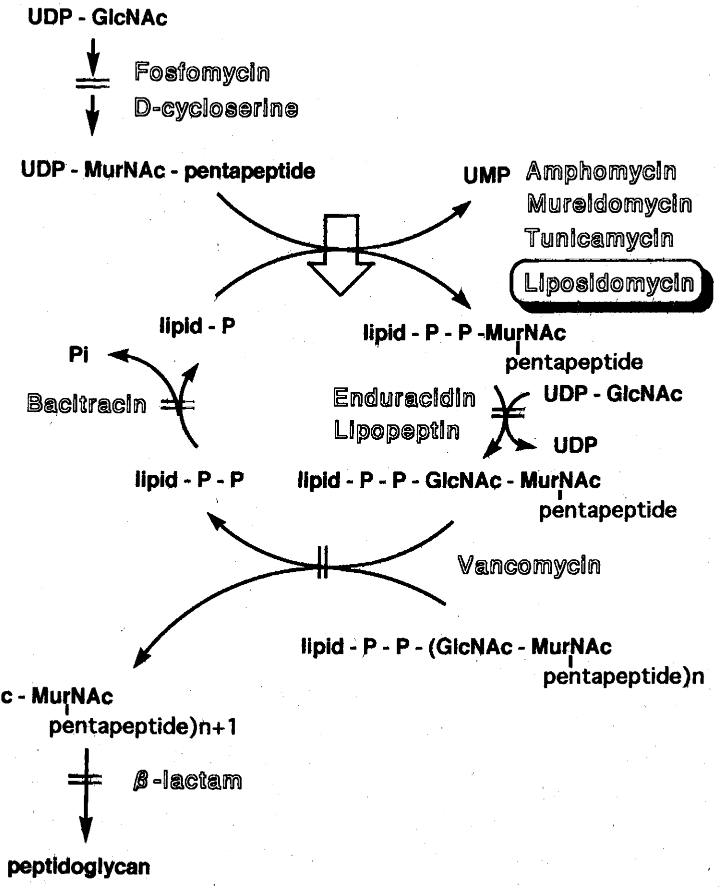
We examined the inhibitory activity against bacterial peptidoglycan biosynthesis, mammalian glycoprotein biosynthesis and growth of BALB/3T3 cells of four different types of liposidomycins which have the structure with or without sulfate and/or 3-methylglutaric acid moieties. Liposidomycins inhibited peptidoglycan biosynthesis about 30~500 times more effectively than tunicamycin, whereas liposidomycins inhibited mammalian glycoprotein biosynthesis about 30~300 times less effectively than tunicamycin. When the cytotoxic effect of liposidomycins and tunicamycin on the growth of mammalian cells were compared, liposidomycins did not show toxicity against BALB/3T3 cell at 25 μg/ml, though tunicamycin inhibited cell growth by 50% at 0.05 μg/ml. On the basis of these results, it is concluded that liposidomycins are selective antibiotics showing highly specific inhibition toward bacterial peptidoglycan biosynthesis.

Many commercial antibiotics including β-lactams, vancomycin *etc.* inhibit the biosynthesis of the cell wall peptidoglycan. The resistance to current antibiotics has caused clinical problems and has emphasized the need to identify new targets for antibiotic action[1,2]. Liposidomycins are unique chemicals that mimic the UDP-MurNAc-pentapeptide-PP-lipid of intermediate in cell wall peptidoglycan biosynthesis[3,4]. Thus liposidomycins A, B and C inhibit peptidoglycan biosynthesis of *Escherichia coli* at $IC_{50} = 0.03$ μg/ml by means of the paper chromatographic method[5]. The primary inhibition site was determined to be phospho-*N*-acetylmuramoyl-pentapeptide-transferase (EC 2.7.8.13, designated as translocase I), the first step in the lipid cycle of peptidoglycan biosynthesis (Fig. 1)[6]. Recently, the inhibition mechanism against translocase I was examined in detail by comparison of liposidomycin B, tunicamycin[7] and mureidomycin A[8]. In this study, liposidomycin B and mureidomycin A were shown to act as slow-binding inhibitors, whereas tunicamycin has a reversible inhibition mechanism[9]. Moreover liposidomycin B inhibited formation of lipid intermediates in glycoconjugate biosynthesis at high concentrations compared with its activity against translocase I[10]. Though liposidomycins are specific inhibitors of translocase I, they do not possess potent antimicrobial activities. It was considered that permeability of liposidomycin into cell membrane would be limited due to the presence of a hydrophilic ionic sulfate moiety. In preceding papers, we have isolated new types of liposidomycins which have a structure without sulfate moiety and show more potent antimicrobial activity than the original types of liposidomycins containing sulfate moiety[11,12].

In this paper, we report the biological activities of four new types of liposidomycins that have the structure with or without sulfate and/or 3-methylglutaric acid

Fig. 1. Schematic of peptidoglycan biosynthesis and site of inhibition of known compounds.



moieties, as compared to tunicamycin.

Materials and Methods

Materials

Liposidomycins were isolated as described[12]. They can be classified into four types according to their structure especially when based on sulfate and 3-methylglutaric acid moieties. [^{14}C]UDP-GlcNAc (251 μCi/mmol) and [^{3}H]UDP-GlcNAc (14.7 Ci/mmol) were purchased from Amersham and NEN, and tunicamycin was from Sigma (T-7765). Other chemicals used were commercially available. The structure of new types of liposidomycins used in this study and tunicamycin were shown in Fig. 2.

Assay of Peptidoglycan Biosynthesis

Assay of peptidoglycan biosynthesis was performed by a slightly modified version of the method previously reported[6]. UDP-MurNAc-pentapeptide and particulate enzyme was prepared from *Bacillus cereus* T and *Escherichia coli* AB 1151. A reaction mixture (50 μl) containing 0.1 M Tris-HCl (pH 7.5), 20 mM $MgCl_2$, 0.1 mM UDP-MurNAc-pentapeptide, 0.01 μCi of [^{14}C]-UDP-GlcNAc, 5 μl of sample and particulate enzyme (15 mg protein/ml) was incubated for 60 minutes at 37°C. After incubation, the reaction mixture was added into 1 ml of cold 5% TCA and filtered through a Whatmann GF/C glass filter. The filter was washed with 5% TCA two times and the remaining radioactivity was counted by an Aloka liquid scintillation counter with a toluene-based scintillation fluid.

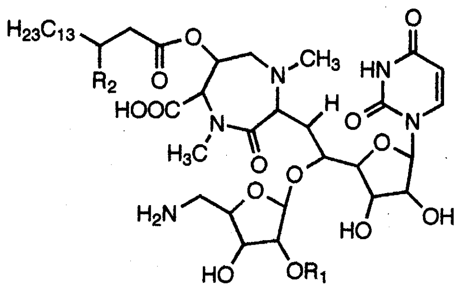
Assay of Dol-PP-GlcNAc Biosynthesis

For the assay of dolichyl-pyrophosphoryl-*N*-acetylglucosamine (Dol-PP-GlcNAc) formation, microsomes were prepared from rat livers. A reaction mixture containing microsomes (4 mg protein/ml), 10 mM Tris-malate buffer (pH 7.1), 0.1 M KCl, 5 mM $MnCl_2$, 5 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 0.1% Triton X-100, 0.1 μCi of [^{3}H]UDP-GlcNAc per ml, and various concentration of test compounds was incubated at 28°C for 8 minutes. Labeled lipids were then extracted and processed as described previously[13]. The radioactivity was counted

Fig. 2. Structures of four types of liposidomycins and tunicamycin.

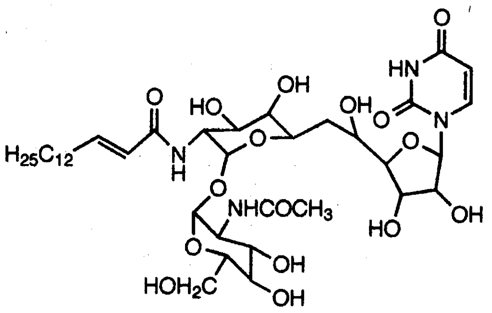
Liposidomycin A-(I) is identical with the original compound of liposidomycin A. Tunicamycin V is an abundant compounds in tunicamycins.

(A) Liposidomycin



	R_1	R_2
A-(I)	SO_3H	$C_6H_9O_4$
A-(II)	SO_3H	H
A-(III)	H	$C_6H_9O_4$
A-(IV)	H	H

(B) Tunicamycin V



by an Aloka liquid scintillation counter with a toluene-based scintillation fluid.

Assay of Cytotoxicity

BALB/3T3 cells were suspended in Dulbecco-modified Eagle's medium (5×10^5 cells/ml) containing 10% fetal bovine serum, plated into 96-well microplates, and cultured in a humidified atmosphere of 95% air-5% carbon dioxide at 37°C. After 24 hours, sample compounds dissolved in methanol were added, and the cells were incubated for an additional 72 hours. The number of viable cells was then determined using the MTT method[14].

Assay of Antimicrobial Activity

Antimicrobial activity was measured using the conventional paper disc method.

Results

Inhibition of Peptidoglycan Biosynthesis

Peptidoglycan inhibitory activity of typical four types of liposidomycins (A-(I), A-(II), A-(III) and A-(IV)) and tunicamycin were shown in Table 1. Inhibitory potencies were recognized as A-(I) > A-(III) > A-(IV) > A-(II). Inhibitory potencies of those liposidomycins were 30~500 times more potent than that of tunicamycin. The 3-methylglutaric acid moiety therefore has an important role for peptiodoglycan inhibition.

Inhibition of Dol-PP-GlcNAc Biosynthesis

Dol-PP-GlcNAc biosynthesis inhibitory activity of typical four types of liposidomycins (A-(I), A-(II), A-(III) and A-(IV)) and tunicamycin were shown in Fig. 3. IC_{50}s of them were 8.4, 4.9, 1.0, 1.8 and 0.029 μg/ml, respectively. The order of inhibition was A-(III) = A-(IV) > A-(II) = A-(I). Inhibitory potencies were 30~300 times less than that of tunicamycin. Contrary to the case of peptidoglycan inhibition, the sulfate moiety in the molecule appears to have an important role for Dol-

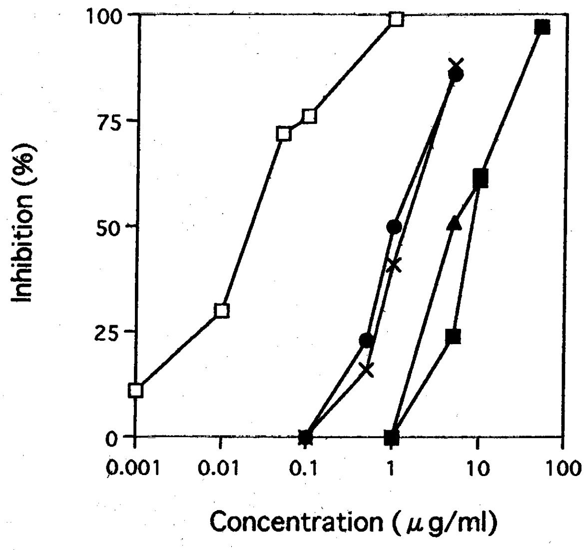
Table 1. Inhibition of peptidoglycan biosynthesis by four types of liposidomycins and tunicamycin.

Compound	Conc. (μg/ml)	Count (dpm)	Inhibition (%)
Control		1442	0
A-(I)	0.01	598	59
	0.1	336	77
	1	295	80
A-(II)	0.01	1288	11
	0.1	663	54
	1	417	71
A-(III)	0.01	766	47
	0.1	420	71
	1	351	76
A-(IV)	0.01	1047	27
	0.1	482	67
	1	401	72
Tunicamycin	1	1004	30
	10	498	65
	100	309	79

Radioactivity incorporated into the TCA-insoluble fraction was determined as described in Materials and Methods. Inhibition (%) was calculated by the equation of (1 − (compoud (dpm) / control (dpm))) × 100.

Fig. 3. Glycoprotein biosynthesis inhibition of four types of liposidomycins and tunicamycin.

Inhibition (%) was calculated by the equation of (1 − (compoud (dpm) / control (dpm))) × 100.
■: liposidomycin A-(I), ▲: liposidomycin A-(II), ●: liposidomycin A-(III), ×: liposidomycin A-(IV), □: tunicamycin.



Radioactivity incorporated into the dolichyl-PP-GlcNAc was obtained by solvent extract and column chromatography as described in the literature[13].

Table 2. Anti-*Mycobacterium* activities of four types of liposidomycins and tunicamycin.

	(I)	(II)	(III)	(IV)	TM
Inhibition zone against *Mycobacterium phlei* IFO 3158 (2 μg/disc, mm)	0	0	14.3	23.4	0

(I)~(IV): Liposidomycins A-(I)~A-(IV), TM: tunicamycin.
Diameter of each inhibition zone was measured.

PP-GlcNAc biosynthesis.

Cytotoxic Activity

Cytotoxic activities of typical four types of liposidomycins (A-(I), A-(II), A-(III) and A-(IV)) and tunicamycin against BALB/3T3 cells were investigated. In contrast to the IC_{50} of 0.05 μg/ml for tunicamycin, all tested liposidomycins did not inhibit the growth of BALB/3T3 cells even at 25 μg/ml.

Antimicrobial Activity

Antimicrobial activity of four types of liposidomycins (A-(I), A-(II), A-(III) and A-(IV)) and tunicamycin against *Mycobacterium phlei* IFO3158 tested at 2 μg/disc are shown in Table 2. Liposidomycins A-(III) and A-(IV) (nonsulfate type) displayed greater antimicrobial activity than liposidomycins A-(I) and A-(II) (sulfate type). At the same concentration of 2 μg/disc, tunicamycin did not show antimicrobial activity against *Mycobacterium phlei* IFO3158. Antimicrobial activities of liposidomycin C-(III) and tunicamycin against various microorganism were shown in Table 3. Tunicamycin inhibited the growth of yeast and fungi, but liposidomycin C-(III) showed only antibacterial activity. Whereas liposidomycin C-(III) inhibited the growth of *Staphylococcus aureus* multi resistant, *Escherichia coli* BE 1186 and *Mycobacterium phlei* IFO 3158, tunicamycin did not show high potency against those strains. The same result was obtained by using liposidomycin M-(III) (this is another abundant compound, data not shown).

Table 3. Antimicrobial activity against various microorganisms of liposidomycin C-(III) and tunicamycin.

Microorganism	Inhibition zone (mm)	
	Liposidomycin C-(III)	Tunicamycin
Escherichia coli AB 1157	0	0
Escherichia coli BE 1186	17.1	0
Escherichia coli multi resistant	0	0
Salmonella typhimurium TV 119	0	0
Salmonella typhimurium SL 1102	(10.9)	0
Pseudomonas aeruginosa IFO 13130	0	0
Pseudomonas aeruginosa N-10 (L-form)	0	0
Staphylococcus aureus IFO 12732	+	13.9
Staphylococcus aureus multi resistant	11.7	0
Bacillus subtilis rec$^+$	13.5	20.6
Bacillus subtilis rec$^-$	14.7	21.2
Micrococcus luteus IFO 12708	0	+
Mycobacterium phlei IFO 3158	34.3	10.6
Xanthomonas oryzae IFO 3312	0	0
Xanthomonas citri IFO 3781	0	0
Erwinia carotovora IFO 12380	0	0
Alternaria mali IFO 8984	0	+
Aspergillus fumigatus IFO 9733	0	0
Botryotinia fuckeliana IFO 5365	0	(13.3)
Glomerella lagenaria IFO 7513	0	0
Pyricularia oryzae IFO 5994	0	(13.3)
Pusarium oxysporum IFO 9761	0	0
Trichophyton rubrum IFO 6203	0	0
Candida albicans IFO 1594	0	+
Schizosaccharomyces pombe IFO 0638	0	17.2
Chlorella vulgaris	0	21.2

All microorganisms were grown in the optimum conditions and diameter of each inhibition zone was measured.

20 μg/disc, ( ): partial inhibition zone.

Discussion

Liposidomycins act as potent and selective inhibitors of bacterial translocase I, which catalyzes the first step in the membrane cycle of bacterial cell wall peptidoglycan biosynthesis. Although it was reported that tunicamycin[7)], amphomycin[15)] and mureidomycin[8)] also inhibited translocase I, this target remains unexploited for therapeutic antibiotics (Fig. 1).

Though liposidomycins are fascinating antibiotics in structure and in specific translocase I inhibitory activity, the antimicrobial activity was not strong enough in the original type (I) compounds[5)]. We isolated new types of liposidomycins with more potent antimicrobial activity[11)]. The nonsulfate type (type (III) and (IV)) had potent antimicrobial activity[12)]. We examined the inhibition activity of all four types of liposidomycins against peptidoglycan biosynthesis, glycoprotein biosynthesis and cell growth. Though type (III) and (IV) had almost the same or less potency as type (I) and (II) against peptidoglycan biosynthesis *in vitro*, they had improved antimicrobial activity against bacteria, especially *Mycobacterium phlei* IFO3158. This indicated that type (III) and (IV) might be able to penetrate through the cell membrane and inhibit the translocase I located in the inner cytoplasmic membrane.

Tunicamycin is also a nucleoside antibiotic which has uracil, tunicamine, GlcNAc and lipid side chains (Fig. 2.)[16)]. Though it inhibited the same site of bacterial peptidoglycan biosynthesis as liposidomycin, it inhibited the mammalian glycoprotein biosynthesis more strongly (Fig. 3.)[17~19)]. Whereas liposidomycin had structural similarity to tunicamycin (Fig. 2.), it inhibited peptidoglycan biosynthesis more potently than glycoprotein biosynthesis. Unique structural features, such as the amino sugar of liposidomycin, 5-amino-5-deoxyribose and/or perhydro-1,4-diazepine moieties might be involved with this specific biological activitiy against peptidoglycan biosynthesis. These results indicated that liposidomycin is a highly potent and selective inhibitor of bacterial peptidoglycan biosynthesis and could lead to the design of useful clinical antibiotic agents.

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