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Antibacterial Activity of Ultrashort Cationic Lipo-β-Peptides[▽]

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Previously reported D,L-lipo-α-peptides and their lipo-β-peptide counterparts (C16-KGGK, C16-KAAK, C16-KKKK, and C12-KLLK) were studied, and the lipo-β-peptides were found to retain antimicrobial activity. Likewise, no significant changes in antimicrobial activity were found upon activity comparisons with D,L-amino acid-based lipopeptides or any L-amino acid lipopeptides. As a defined amphipathic structure is unlikely to form with such short molecules and as similar activities were obtained from all lipopeptides, we suspect that the action of membrane permeation is retained.

The rise of antibiotic-resistant microbes has prompted interest in novel therapeutics with new modes of action, including antimicrobial lipopeptides. Naturally occurring lipopeptides produced by bacteria, yeasts, and fungi with largely antifungal activity exist, but some also show antibacterial activity (2, 5, 7). Native lipopeptides are cyclic and anionic and contain short peptide portions of six or seven D- and L-amino acids that are toxic to mammalian cells due to a lack of selectivity (3, 10). However, studies of synthetic lipopeptides formed from acylated antimicrobial peptides report a marked improvement in bioactivity against bacteria (1, 4, 6). Recently, a series of short lipopeptides were synthesized from biologically inactive D,L cationic tetrapeptides and found to possess cell-lysing activity against a variety of gram-positive and gram-negative bacteria, with both aliphatic chain length and peptide sequence determining cell type selectivity (7). This study aims to investigate the biological activity of short cationic lipo-β-peptides on the basis of previously reported lipo-α-peptides (7). As with incorporation of D-enantiomers, peptidomimetics incorporating β-amino acids offer the potential benefit of metabolic and enzymatic stability against proteases, one of the major drawbacks in peptide-based drug development (11).

American Type Culture Collection (ATCC) strains as well as clinical isolates from the Canadian Intensive Care Unit (CAN-ICU) study were used, including *Staphylococcus aureus* ATCC 29213, methicillin-resistant *Staphylococcus aureus* ATCC 33592, *Staphylococcus epidermidis* ATCC 14990, methicillin-resistant *Staphylococcus epidermidis* (MRSE) (cefazolin MIC, >32 μg/ml) CAN-ICU 61589, *Enterococcus faecalis* ATCC 29212, *Enterococcus faecium* ATCC 27270, *Streptococcus pneumoniae* ATCC 49619, *Escherichia coli* ATCC 25922, *E. coli* (gentamicin-resistant) CAN-ICU 61714, *E. coli* (amikacin MIC, 32 μg/ml) CAN-ICU 63074, *Pseudomonas aeruginosa* ATCC 27853, *P. aeruginosa* (gentamicin-resistant) CAN-ICU 62308, *Stenotrophomonas maltophilia* CAN-ICU 62584, *Acinetobacter baumannii* CAN-ICU 63169, and *Klebsiella pneumoniae* ATCC 13883 (13).

Both the lipo-α-peptides and the lipo-β-peptides (Table 1) investigated in this study were synthesized by solid-phase peptide synthesis using standard 9-fluorenylmethoxy carbonyl chemistry on Rink amide-4-methylbenzhydrylamine hydrochloride salt resin. Palmitic acid and lauric acid were conjugated to the tetrapeptides via modified solid-phase methods. TBTU [2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate] (3 eq), lipophilic acid (3 eq), and diisopropylethylamine (9 eq) reacted in a solution of 45% CH_2Cl_2 in dimethylformamide, and the process was repeated twice. Lipopeptide cleavage in 95% trifluoroacetic acid was achieved, followed by purification on reversed-phase C_{18} silica. The homogeneity and identity of the synthetic peptides were assessed by electrospray ionization-mass spectrometry, ^{1}H nuclear magnetic resonance, and ^{13}C nuclear magnetic resonance.

Antibacterial activity against gram-positive and gram-negative microorganisms was investigated via broth macrodilution tests using CLSI methodology (13). Stock solutions of lipopeptide antibiotics in water were brought to a standard concentration of 512 μg/ml, with only βC12-KLLK and βC16-KAAK requiring a minute amount of dimethyl sulfoxide. Organisms were subcultured and isolated on blood agar, suspended in 3 ml of Mueller-Hinton broth at the turbidity of a 0.5 M McFarland standard, and diluted to approximately 10^5 CFU/ml before introduction into tubes containing serially diluted lipopeptide antibiotic in Mueller-Hinton broth. Testing of activity against *S. pneumoniae* used broth supplemented with laked horse blood to give 5% horse blood in experimental tubes. The turbidity resulting from the lipopeptide solution in broth required the creation of control tubes lacking microbes serving as turbidity controls. All tubes were incubated overnight for 16 to 20 h at 37°C. Colony counts for a diluted 10^5-CFU/ml solution of microorganisms confirmed the validity of the trial, with colony counts expected in the 10^5-CFU/ml range with incubation overnight in a CO_2 incubator at 37°C and 5% CO_2.

In this study, a total of 12 lipopeptides were synthesized with a tetrapeptide moiety containing (i) all L-amino acids, (ii) D,L-amino acids, and (iii) all β-amino acids, based on the following four sequences: C16-KGGK, C16-KAAK, C16-KKKK, and C12-KLLK. These sequences are based on a representative sample of the highly active N-terminal acylated lipopeptides reported by Makovitzki and coworkers (7), and as such, the

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TABLE 1. Antimicrobial activities of ultrashort cationic lipopeptides[a]

Control organism	MIC (μg/ml)												
	Gentamicin	αC16-KGGK	αC16-KKKK	αC16-KAAK	αC12-KLLK	αC16-KGGK	αC16-KKKK	αC16-KAAK	αC12-KLLK	βC16-KGGK	βC16-KKKK	βC16-KAAK	βC12-KLLK
S. aureus ATCC 29213	1	8	32	16	16	16	16	8	16	16	16	64	32
MRSA ATCC 33592	2	16	16	16	16	16	16	32	16	32	16	32	32
S. epidermidis ATCC 14990	0.25	4	4	8	16	8	4	4	8	8	4	8	16
MRSE CAN-ICU 61589	32	8	8	8	16	8	8	8	16	16	4	16	32
E. faecalis ATCC 29212	ND	8	16	16	32	16	32	16	32	32	32	32	64
E. faecium ATCC 27270	ND	16	16	8	16	16	16	8	32	32	16	32	32
S. pneumoniae ATCC 49619	4	128	>64	128	128	128	>32	128	64	128	128	>64	128
E. coli ATCC 25922	1	16	16	16	128	16	32	32	64	32	16	64	64
E. coli CAN-ICU 61714	128	16	16	16	128	64	32	32	64	32	32	64	64
E. coli CAN-ICU 63074	8	16	32	16	128	16	32	32	64	32	32	64	64
P. aeruginosa ATCC 27853	8	64	64	32	128	64	32	64	128	64	64	256	128
P. aeruginosa CAN-ICU 62308	128	64	256	64	128	64	256	64	128	>64	128	128	128
S. maltophilia CAN-ICU 62584	>512	128	256	128	>256	64	256	128	>256	256	256	>128	256
A. baumannii CAN-ICU 63169	128	128	256	128	>64	64	256	128	>256	128	256	256	>128
K. pneumoniae ATCC 13883	0.25	64	256	128	256	64	256	128	>64	128	128	>128	256

[a] Underlined letters represent the positions of the D-enantiomers. MRSA, methicillin-resistant *S. aureus*; ND, not determined.

D,L-amino acid-based lipopeptides serve as the control group. The sequences for the lipo-α-peptides and lipo-β-peptides are listed in Table 1, with the positions of the D-enantiomers shown.

As studies previously indicated, the lipopeptides containing only L-amino acids did not show significant differences in antimicrobial activity from peptides incorporating the D-enantiomer of an amino acid (8). Also, the activities of the lipo-β-peptides were comparable to those of their D,L-amino acid counterparts, with limited differences (almost all values within a twofold dilution) (Table 1). Gram-positive organisms proved generally more susceptible to these lipopeptide agents than did gram-negative bacteria. Among gram negatives, only *E. coli* strains proved somewhat susceptible to all sequences of lipopeptides, although the MICs were higher with the C12-KLLK series, in which MICs ranged between 64 and 128 μg/ml. Interestingly, among gram positives, only *S. pneumoniae* proved less susceptible to the lipopeptide antibiotics, with MICs largely greater than 64 μg/ml. However, it should be stated that the MICs for *S. pneumoniae* were reduced 8- to 32-fold for all lipopeptides when the MIC experiments were performed with Todd Hewitt instead of Mueller-Hinton broth supplemented with laked horse blood. This suggests that lipopeptides are highly protein bound.

Among all species tested, *S. epidermidis* consistently showed the highest levels of susceptibility to all synthesized lipopeptides, followed closely by its antibiotic-resistant counterpart, MRSE. Likewise, all other organisms for which antibiotic-resistant strains were tested showed activities similar to those of their nonresistant counterparts. Organisms such as *S. aureus*, *E. coli*, and *P. aeruginosa* had MICs that, for the most part, did not vary over more than a twofold dilution. The organisms *S. maltophilia*, *A. baumannii*, and *K. pneumoniae* proved least susceptible to all lipopeptides.

Since resistance to lipopeptides is a generally rare occurrence (12), and because of the advantages that β-amino acids provide (9, 11), lipo-β-peptides merit further work as potential novel therapeutics. Our results demonstrate that lipo-β-peptides display antimicrobial activities comparable to those of lipo-α-peptides. Previous studies have shown that the mode of action of ultrashort α-lipopeptides involves permeation and disintegration of membranes, similar to what was found for many long antimicrobial peptides (7). This mode of action makes it difficult for the microorganisms to develop resistance. It is unlikely that ultrashort α- and β-lipopeptides as used in this study will form a defined and stable amphipathic structure. This implies that ultrashort α- and β-lipopeptides will retain similar modes of antibacterial action.

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