Antimicrobial Lipopolypeptides Composed of Palmitoyl Di- and Tricationic Peptides: *In Vitro* and *in Vivo* Activities, Self-Assembly to Nanostructures, and a Plausible Mode of Action[†]

Arik Makovitzki, Jonathan Baram, and Yechiel Shai*

Department of Biological Chemistry, The Weizmann Institute of Science, Rehovot, 76100 Israel

Received June 22, 2008; Revised Manuscript Received August 6, 2008

ABSTRACT: Antimicrobial lipopeptides are produced nonribosomally in bacteria and fungi during cultivation. They are composed of a cationic or an anionic peptide covalently bound to a specifically modified aliphatic chain. Most of the peptidic moieties have complex cyclic structures. Here we report that conjugation of a palmitic acid to the N-terminus of very short cationic di- and tripeptides composed of all L- and D,L-amino acids endowed them with potent antimicrobial activities. Interestingly, cell specificity was determined by the sequence of the short peptidic chain. Palmitoyllysine served as a control and was inactive toward all microorganisms tested. Replacing an L-amino acid with its D-enantiomer did not affect the activity of the corresponding lipopeptides. Importantly, selected lipopeptides were also potent *in vivo* in a mouse model of *Candida albicans* infection. Bacterial leakage experiments and negative staining electron microscopy suggest that their mode of action involves permeation and disintegration of the microorganism's membrane, similar to many long antimicrobial peptides and lipopeptides. Interestingly, each lipopeptide assembled in solution into a nanostructure with a unique morphology which could partially explain differences in their biological activity. Besides adding important information on the parameters necessary for antimicrobial lipopeptides to kill microorganisms, the simple composition of these minilipopeptides and their diverse cell specificities make them attractive candidates for various applications.

Antimicrobial peptides (AMPs)[1] belong to a diverse and evolutionary old group of molecules that are important effectors of innate immunity. AMPs are employed as the first line of defense against invading microorganisms by most known organisms (*1*−*4*). Many AMPs physically and rapidly permeate and destroy the cell membrane of microorganisms, causing damage that is difficult to fix. Therefore, microbial resistance may occur with a low probability (*4*). Structurally, AMPs are 12−50 amino acids long, carry a net positive charge, and are composed of ≈50% hydrophobic amino acids (*5, 6*). Another group of antimicrobial agents includes native lipopeptides that are produced nonribosomally in bacteria and fungi during cultivation on various carbon sources (*7*−*11*). They are composed of aliphatic acid attached to the N-terminus of a short cationic or anionic peptidic moiety of six to seven amino acids. Most native lipopeptides have complex cyclic structures (*2, 7, 12*). The mode of action of some of them is via perturbation of the cell membrane of the microorganism, thus affecting the transmembrane electric potential (*11*−*24*). A major step in the activity of cationic lipopeptides is their initial binding to the negatively charged lipopolysaccharide (LPS) of Gram-negative bacteria or to the lipotechoic acid of Gram-positive bacteria. The lipopeptides traverse into the inner phospholipid membrane, which is highly enriched with phosphatidylglycine (PG), and permeate the membrane (*2, 12*). In fungi, the lipopeptides bind to the negatively charged membrane phosphatidylinositol (PI) (*7, 8, 25*) and to the negatively charged terminal sialic acid moieties (*26, 27*). Some of these molecules are highly active against bacteria including multiresistant strains (*17*−*19, 28, 29*). Others display solely antifungal activity (*7, 8, 30*) and a few both antifungal and antibacterial activity (*8, 31*). Several members of this family of native antimicrobial lipopeptides and derivatives were approved for clinical use by the Food and Drug Administration (FDA). These include daptomycin, which is active only toward Gram-positive bacteria, polymyxin B, which is active only toward Gram-negative bacteria, and echinocandins, which are β-1,3-D-glucan synthase inhibitors and therefore are active only toward fungi.

Previous studies have shown that conjugation of a fatty acid to linear AMPs results in increased antimicrobial activity (*11, 20*−*22, 32*). Furthermore, fatty acid conjugation to nonactive cationic peptides endowed them with antimicrobial capacity (*23, 33*−*35*). Interestingly, recent studies have shown that fatty acids can compensate for the length of the peptidic chain (*24, 33, 36*−*40*). In particular, we have found that the attachment of fatty acids to otherwise inert cationic tetrapeptides endowed them with *in vitro* potent

[†] This study was supported by the Pasteur-Weizmann Joint Research Program. Y.S. has the Harold S. and Harriet B. Brady Professorial Chair in Cancer Research.

* To whom correspondence should be addressed. Tel: 972-8-9342711. Fax: 972-8-9344112. E-mail: Yechiel.Shai@weizmann.ac.il.

[1] Abbreviations: AMPs, antimicrobial peptides; BHA, 4-methylbenzhydrylamine resin; Fmoc, 9-fluorenylmethoxycarbonyl; CFU, colony-forming units; hRBC, human red blood cells; MIC, minimal inhibitory concentration; PBS, phosphate-buffered saline; PC, egg phosphatidylcholine; PE, *E. coli* phosphatidylethanolamine; PG, egg phosphatidylglycerol; TFA, trifluoroacetic acid.

10.1021/bi8011675 CCC: $40.75
Published on Web 09/11/2008

activity against various microorganisms including antibiotic-resistant strains (*24*). Interestingly, lipopolyamines have been shown to mimic such short lipopeptides and were endowed with potent antibacterial activity and defined specificities (*41, 42*). In addition, these lipopeptides and polyamines mimic partially quaternary ammonium salts (alkanoylcholine or chemical surfactants), which constitute a broad class of metabolites commonly occurring in nature. However, in contrast with the lipopeptides and lipoamines, the alkanoylcholines are toxic to all types of cells (*43*).

Here we report on the synthesis and antimicrobial activity *in vitro* and in an animal skin model of fungal infection of very short cationic lipopeptides composed of a palmitic acid attached covalently to all L- and D,L-tri- and dipeptides. To shed light on their plausible mode of action, we studied their ability to perturb the microorganism's membrane, to induce cell wall lysis, and to assemble into nanostructures in solution. The results are discussed with regard to the parameters involved in the potency and cell specificity of this new family of cationic minilipopeptides and their potential to be developed as antimicrobial agents.

MATERIALS AND METHODS

Materials. Rink amide MBHA resin, 4-methylbenzhydrylamine resin (BHA), and 9-fluorenylmethoxycarbonyl (Fmoc) amino acids were obtained from Calbiochem-Novabiochem AG (Switzerland). Lauric acid (dodecanoic acid) was purchased from Sigma Chemical Co. (Israel). Other reagents used for peptide synthesis included trifluoroacetic acid (TFA; Sigma), piperidine (Merck), *N*,*N*-diisopropylethylamine (DIEA, Sigma), *N*-methylmorpholine (NMM, Fluka), *N*-hydroxybenzotriazole hydrate (HOBT; Aldrich), 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), and dimethylformamide (DMF, peptide synthesis grade; Biolab). Phosphatidylcholine (PC, from egg yolk), phosphatidylethanolamine (PE, from *Escherichia coli*), phosphatidylinositol (PI, from bovine liver), egg phosphatidylglycerol (PG), and ergosterol were purchased from Sigma. Cholesterol (extra pure) was supplied by Merck (Darmstadt, Germany). Calcein was purchased from Molecular Probes (Junction City, OR). All other reagents were of analytical grade. Buffers were prepared in double-distilled water. Amphotericin B and gentamicin were purchased from Sigma Chemical Co. (Israel). RPMI 1640 was purchased from Biological Industries (Beit Haemek, Israel).

Peptide Synthesis, Acylation, and Purification. Peptides were synthesized by a 9-fluorenylmethoxylcarbonyl (Fmoc) solid-phase method on Rink amide MBHA resin by using a ABI 433A automatic peptide synthesizer. The lipophilic acid was attached to the N-terminus of a resin-bound peptide by standard Fmoc chemistry followed by peptide cleavage from the resin and purification by RP-HPLC (>98%) (*21*). The composition of the lipopeptides was confirmed by electrospray mass spectroscopy and amino acid analysis.

Antifungal Activity. The antifungal activity of the lipopeptides was measured using the conditions of the National Committee for Clinical Laboratory Standards document M27-A. The peptides were examined in sterile 96-well plates (Nunc F96 microtiter plates) in a final volume of 200 μL as follows: 100 μL of a suspension containing fungi at a concentration of 2×10^3 colony-forming units/mL in culture medium (RPMI 1640, 0.165 M MOPS, pH 7.4, with L-glutamine, without $NaHCO_3$ medium) was added to 100 μL of water containing the peptide in serial 2-fold dilutions. The fungi were incubated for 24 h for *Aspergillus fumigatus* (ATCC 26430) and *Aspergillus flavus* (ATCC 9643) and for 48−72 h for *Candida albicans* (ATCC 10231) and *Cryptococcus neoformas* (ATCC MYA-422) using a Binder KB115 incubator. Growth inhibition was determined by measuring the absorbance at 620 nm in a microplate autoreader El309 (Biotek Instruments). Antifungal activities are expressed as the minimal inhibitory concentration (MIC), the concentration at which no growth was observed.

Antibacterial Activity. The antibacterial activity of the lipopeptides was examined in sterile 96-well plates (Nunc F96 microtiter plates) in a final volume of 100 μL as follows: aliquots (50 μL) of a suspension containing bacteria at a concentration of 10^6 colony-forming units/mL in culture medium were added to 50 μL of water containing the peptide (prepared from a stock solution of 1 mg/mL peptide in water) in serial 2-fold dilutions in LB. Inhibition of growth was determined by measuring the absorbance at 492 nm with a Microplate autoreader El309 (Biotek Instruments) after an incubation of 18−20 h at 37 °C. Antibacterial activities were expressed as the MIC, the concentration at which no growth was observed after 18−20 h of incubation. The bacteria used were *E. coli* ATCC 25922, *E. coli* ATCC D21, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 6538P, and gentamicin-resistant *A. baumannii*.

Hemolysis of Human Red Blood Cells (hRBCs). The assay was done by using a protocol described previously (*44*), with a final volume of 100 μL of PBS solution containing the lipopeptides and hRBCs (final concentration 4%). Release of hemoglobin was monitored by measuring the absorbance of the supernatant at 540 nm. Controls for 0 hemolysis (blank) and 100% hemolysis consisted of hRBCs suspended in PBS and 1% Triton, respectively.

Transmembrane Potential Depolarization Assay with Bacteria. Bacteria were grown at 37 °C with agitation to midlog phase ($OD_{600} = 0.4$). The cells were centrifuged and resuspended to a OD_{600} of 0.05 in PBS. Next, the cells were incubated with 2 μM $DiIC_1(5)$ with agitation until a stable reduction of fluorescence was achieved (around 4 min), indicating the incorporation of the dye into the bacterial membrane. Membrane depolarization was monitored by observing the change in the intensity of fluorescent emission of the membrane potential-sensitive dye $DiIC_1(5)$ (excitation wavelength $\lambda_{ex} = 638$ nm, emission wavelength $\lambda_{em} = 660$ nm) after the addition of different concentrations of lipopeptides.

SYTOX Green Uptake Assay. C. albicans was grown in potato dextrose broth, whereas *E. coli* and *S. aureus* were grown in LB at 37 °C, washed, and suspended in PBS. Cells were suspended (2×10^7 cells/mL) in 10 mM sodium phosphate buffer (pH 7.4) and were incubated with 1 μM SYTOX green for 15 min with agitation in the dark (*45*). After the addition of the peptides, the increase in fluorescence, owing to the binding of the dye to intracellular DNA, was monitored (excitation wavelength $\lambda_{ex} = 485$ nm, emission wavelength $\lambda_{em} = 520$).

Negatively Staining Electron Microscopy. (i) Visualization of Bacteria and Fungi before and after Treatment with the Lipopeptide. Samples containing *C. albicans* ATCC 10231,

Table 1: Antimicrobial MICs of the Lipopeptides (μM)

	Gram-negative bacteria			Gram-positive bacteria			fungi	
peptide designation	*E. coli* 25922	*P. aeruginosa* 27853	*E. cloacae* 49141	*A. baumannii* 19606	*S. aureus* 25923	*E. faecalis* 19433	*C. albicans* 10231	*A. fumigatus* 26430
C16-KAK	>100	>100	>100	>100	>100	100	>100	>100
C16-KGK	12.5	12.5	100	50	6.2	25	12.5	6.25
C16-KKK	1.56	12.5	3.1	12.5	3.1	6.2	6.2	3.1
C16-K*K*K	1.56	12.5	6.2	12.5	6.2	6.2	6.2	6.2
C16-KLK	>100	>100	100	>100	12.5	25	9.4	9.4
C16-K*K*	12.5	25	50	75	12.5	25	9.4	6.2
C16-K	>100	>100	>100	>100	>100	>100	>100	>100
gentamicin	6.3	9.4	6.3	100	2.3	25		
amphotericinB							1.5	6.3

E. coli ATCC 25922, and *S. aureus* ATCC 6538P (3×10^7 CFU/mL) were incubated with 50 μM lipopeptides dissolved in PBS at their MIC for 15 min and centrifuged at 420*g*. Controls were made in the presence of lipopeptides. The yeasts were fixed by incubation with 1% glutaraldehyde in PBS for 20 min. The pellets were resuspended; a drop containing the fungi was deposited onto a carbon-coated grid and negatively stained with 1% (w/v) uranyl acetate, and the bacteria were negatively stained with 2% (w/v) PTA.

(ii) Visualization of Nanostructures Formed by the Lipopeptides in Solution. Experiments were done at a lipopeptides concentration of 200 μM in double-distilled water. The samples were vortexed and sonicated. A drop containing the lipopeptides was deposited onto a carbon-coated grid and negatively stained with 2% phosphotungstic acid (PTA), pH 6.5.

Grids were examined using a JEOL JEM 100B electron microscope (Japan Electron Optics Laboratory, Tokyo, Japan). In both experiments, the grids were examined using a JEOL JEM 100B electron microscope (Japan Electron Optics Laboratory, Tokyo, Japan).

Mouse Infection, Treatment, and Skin Histology. C. albicans ATCC 36082 (10^8 CFU/mL) complexed to Cytodex beads (Sigma, St. Louis, MO) as a carrier were subcutaneously injected (150 μL) into the dorsal side of 5−6-week-old CD1 nude female mice weighing 20−25 g (Harlen Co., Rehovot, Israel) (*46*). One hour after infection, a 100 μL solution of 0.4 μg/μL lipopeptides (670 μM) dissolved in PBS was sc injected into the infected site. Similarly, during the next 3 days, the lipopeptides were injected sc once a day. On the fifth day, the sites of the infection were biopsied and fixed in 10% buffered formaldehyde. Paraffin-embedded 5-μm sections were stained with PAS (Periodic acid−Schiff) and examined using a microscope (Eclipse E800M; Nikon, Tokyo, Japan) with a digital camera (DXM1200; Nikon, Tokyo, Japan).

RESULTS

We synthesized a series of short all L- and D,L-amino acid peptides linked to palmitic acid. The sequence of the peptidic moiety was KK and KXK (X designates A, G, L, or K, and italic letters designate the D-amino acid enantiomer). Palmitic acid attached to a single lysine served as a control. The sequences of the lipopeptides are shown in Table 1. All of the peptides were amidated at their C terminus.

Biological Activity of the Lipopeptides. The lipopeptides were assayed against representative Gram-positive and Gram-negative bacteria and fungi, as well as against a highly diluted solution [4% (v/v)] of human erythrocytes (Table 1 and Figure 1). The antibiotics gentamicin and amphotericin B served as controls for bacteria and fungi, respectively. The data shown in Table 1 indicate that three amino acids are sufficient to induce antimicrobial activity and cell specificity and that a change of one amino acid altered these biological functions. For example, the lipopeptide C16-KAK was completely inactive toward all tested cells, C16-KLK was inactive only toward Gram-negative bacteria, but it was active against Gram-positive bacteria and fungi. C16-KGK was active toward most bacteria and fungi tested, and C16-KKK was highly potent toward all tested cells. Similar results were obtained when the central lysine was replaced by its D-isomer (Table 1). The lipopeptide C16-K*K*, maintained partial activity but still was potent toward fungi. A single lysine attached to palmitic acid was inactive.

The data also reveal that all of the lipopeptides are nonhemolytic at their MICs, and besides all K-containing lipopeptides, the three others were nonhemolytic up to a concentration of 50 μM.

Permeation of Bacterial and Fungal Cell Membranes by Lipopeptides. To test the ability of the lipopeptides to permeate the bacterial membrane, we used two assays:

(i) We monitored the collapse of the transmembrane potential in living Gram-negative bacteria *E. coli* and Gram-positive bacteria *S. aureus* in the presence of the lipopeptides by using a potential-sensitive dye (Figure 2). The data revealed that all of the peptides are able to induce the collapse of the transmembrane potential, and the order of this activity correlates with their antimicrobial activity. The most active lipopeptides, C16-KKK and C16-KGK, were the most active in this assay, and the less active lipopeptides C16-K and C16-KAK, were the least active in this assay. These results

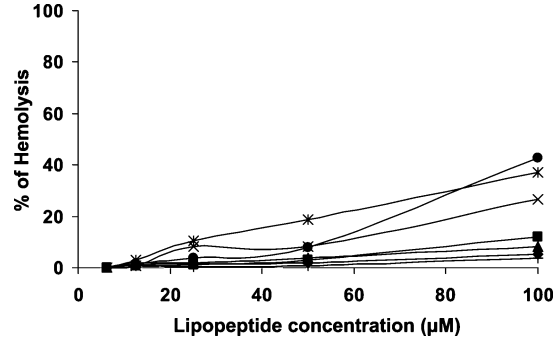

FIGURE 1: Dose response of the hemolytic activity of the lipopeptides toward 4% hRBC. Designations are C16-KAK (◆), C16-KGK (■), C16-KLK (▲), C16-K*K*K (●), C16-KKK (∗), C16-K*K* (×), and C16-K (+).

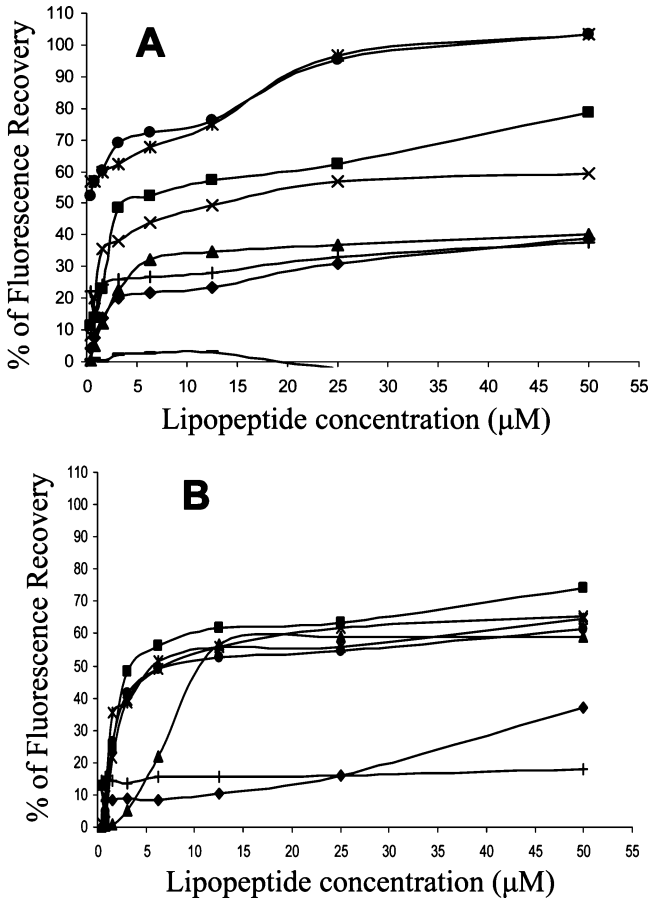

FIGURE 2: Dose-dependent dissipation of the membrane potential in *E. coli* (A) and *S. aureus* (B) after adding the lipopeptides to PBS buffer containing the bacteria preequilibrated with the fluorescent dye $DiIC_1(5)$. Fluorescence recovery was measured for 30 min after the peptides were mixed with the bacteria, with excitation set at 485 nm and emission at 520 nm. The fluorescence increase obtained by using 50 μM melittin was taken as 100%. All readings were normalized by subtracting parasite scattering and the basal fluorescence of the dye. Designations are C16-KAK (◆), C16-KGK (■), C16-KLK (▲), C16-K*K*K (●), C16-KKK (∗), C16-K*K* (×), and C16-K (+).

suggest that their mode of action involves a membranolytic effect. The finding that the two inactive lipopeptides, C16-K and C16-KAK, can partially dissipate the transmembrane potential but do not kill the bacteria is in line with earlier findings that partial collapse of the potential by AMPs is not sufficient to kill the bacteria (*47*).

(ii) We monitored the entrance of the cationic dye SYTOX green (molecular mass 900 Da) into *C. albicans* cells (Figure 3). SYTOX green cannot enter into an intact cell unless its membrane is disrupted by external compounds. Once inside the cell, the florescence of the dye increases drastically owing to its binding to intracellular nucleic acids. Since the entrance of SYTOX requires significant membrane damage, only potent lipopeptides should be active in this assay. Indeed, when *C. albicans* was treated with the lipopeptides, only the biologically active lipopeptides (e.g., C16-KKK, C16-K*K*K, C16-KLK, C16-KGK, and C16-K*K*) were active in this assay, and their order of activity correlates with their antifungal activity. No membranolytic activity was observed with the nonbiologically active C16-KAK and C16-K.

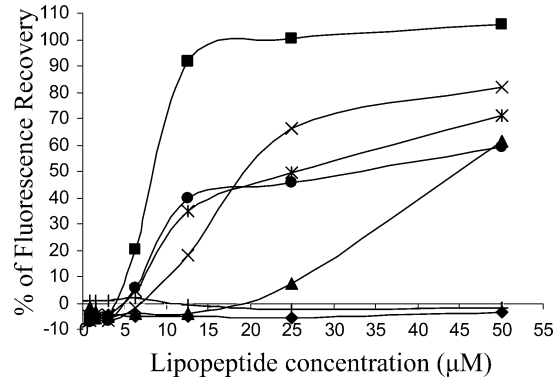

FIGURE 3: Influx of the vital dye SYTOX green into *C. albicans* after adding the lipopeptides. The increase in fluorescence was monitored with excitation set at 485 nm and emission at 520 nm. The fluorescence increase obtained by using 50 μM melittin was taken as 100%. All readings were normalized by subtracting parasite scattering and the basal fluorescence of the dye. Designations are C16-KAK (◆), C16-KGK (■), C16-KLK (▲), C16-K*K*K (●), C16-KKK (∗), C16-K*K* (×), and C16-K (+).

Overall, these two assays and the EM studies (see below) support a membranolytic effect as part of their mode of action.

Visualization of Cell Damage by Using Transmission Electron Microscopy. C. albicans, *S. aureus,* and *E. coli* were treated with different lipopeptides for 15 min at their MICs and visualized by using transmission EM (Figure 4). The images reveal a direct correlation between the biological function of the lipopeptides (Table 1) and their ability to disrupt the cell membrane (Figure 4). For example, the lipopeptide C16-KKK caused a drastic change in the morphology of *E. coli*. Initially *E. coli* swelled up, possibly owing to membrane dysfunction, and its structure changed from rod-shaped to sphere-shaped. Adjacent to it are the remains of other bacteria cells that had collapsed. C16-KKK also caused wide-scale damage to the membranes of *S. aureus* and *C. albicans*. The lipopeptide C16-KGK caused blebs and disrupted large portions of the *E. coli* cell wall and membrane. Moreover, it created massive disruption of the cell wall and membrane of *S. aureus* and *C. albicans* as well. No significant damage to *E. coli* was noted after treatment with C16-KLK in correlation with its inactivity against these bacteria (Table 1). However, C16-KLK damaged *S. aureus* and *C. albicans*, the two bacteria that are sensitive to it. Note also that practically no significant damage was caused to both bacteria and fungi when the inactive C16-KAK was used, although some blebs occurred on the *Candida* surface.

Lipopeptides Form Nanostructures in Solution Visualized by EM. We used negatively staining electron microscopy to visualize whether the lipopeptides can form large oligomers in solution. The data reveal that all the lipotripeptides form nanostructures in solution but with a unique morphology for each one of them (Figure 6). Some form fibrils similarly to amyloids (C16-KGK and C16-KLK), while others have a micelle-like morphology (C16-KAK and C16-KKK). However, magnifications of these structures revealed different types of packing as discussed in detail in the Discussion section.

In Vivo Treatment of C. albicans Infection. CD1 nude mice were infected subcutaneously with *C. albicans* complexed to Cytodex beads (*46*) and treated 1 h after the infection

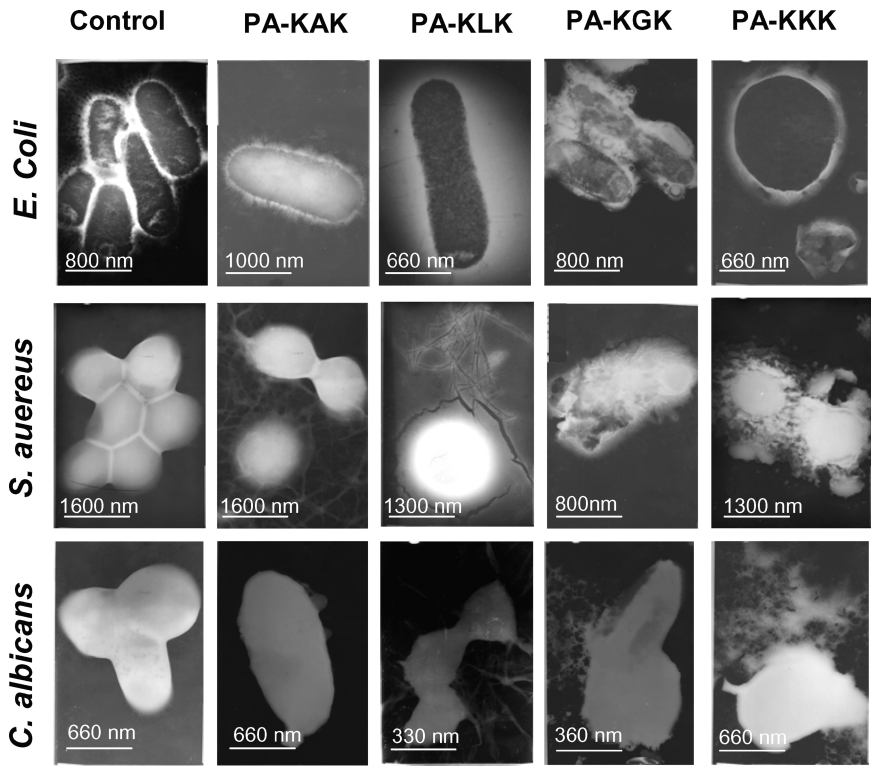

FIGURE 4: Electron micrographs of negatively stained *E. coli* (top), *S. aureus* (middle), and *C. albicans* (bottom) untreated or treated with the lipopeptides. The lipopeptides were used at their MICs.

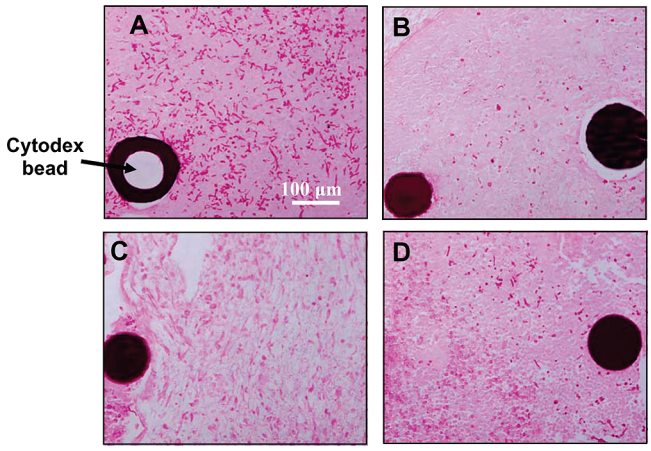

FIGURE 5: Skin from murine dorsal sides infected by subcutaneous injection of *C. albicans* complexed with Cytodex beads treated with vehicle control (A), C16-KGK (B), C16-K*K*K (C), and C16-K*K* was biopsied 5 days after the infection and stained with PAS. *C. albicans* fungal forms are stained in red. Skin tissues treated with vehicle control (A) show evidence of multiple *C. albicans* fungal forms throughout the samples of skin tissues. Skin tissues treated with C16-K*K*K (C) were cleared from fungal forms. Skin tissues treated with C16-KGK (B) and C16-K*K* (D) show a significantly reduced number of fungal forms.

and during the next 3 days with one sc injection each time, at the site of the infection. We used 100 μL of vehicle control or 100 μL of a solution of 0.4 μg/μL lipopeptides C16-K*K*K, C16-K*K*, and C16-KGK. Five days after the infection, skin tissues were removed from the site of the infection for histological examination by PAS. The data shown in Figure 5 revealed severe fungal contamination throughout the skin tissues in the mice that were treated with vehicle control. In contrast, the skin of the mice treated with C16-K*K*K was devoid of fungal contamination. Similarly, only slight fungal contamination was obtained in the skin of mice treated with C16-K*K* and C16-KGK. Importantly, no damage to the skin tissue was observed during the entire experiment.

DISCUSSION

Most native and synthetic cationic antimicrobial peptides are composed of ~12−50 amino acids, whereas antimicrobial lipopeptides are characterized by a shorter peptidic moiety composed of six to seven D- and L-amino acids and usually have a complex cyclic structure attached to a specific aliphatic chain (*2, 7, 8, 10, 12*). The interesting finding in this study is that the attachment of palmitic acid to a cationic peptide, as short as a tripeptide, can compensate for the length of hydrophobicity of the peptidic chain and can endow the resulting lipopeptides with potent antimicrobial activity that is similar to the activity of longer antimicrobial peptides and lipopeptides. Moreover, substituting only one of the triamino acids is sufficient to create molecules with different biological functions, i.e., with a broad spectrum of activity against all strains of bacteria and fungi, or toward Gram-positive bacteria and fungi, but not toward Gram-negative bacteria. On the basis of these results, one can speculate that further substitutions with other amino acids should yield lipopeptides with additional spectra of activities. Note also that the attachment of palmitic acid to two lysines, either all L-amino acids (*40*) or D,L-amino acids (this study) still preserves antimicrobial activity, although with a lower potency and with limited ability for amino acid substitution and, hence, less specificities toward various cells. The finding that a single lysine attached to a palmitic acid is not active supports the notion that the activity of these lipopeptides is not dictated solely by the hydrophobic palmitic chain but also requires a specific sequence. This is further supported by the finding that C16-KAK is not active at all, whereas all other tripeptides tested are active. Furthermore, the hydrophobicity

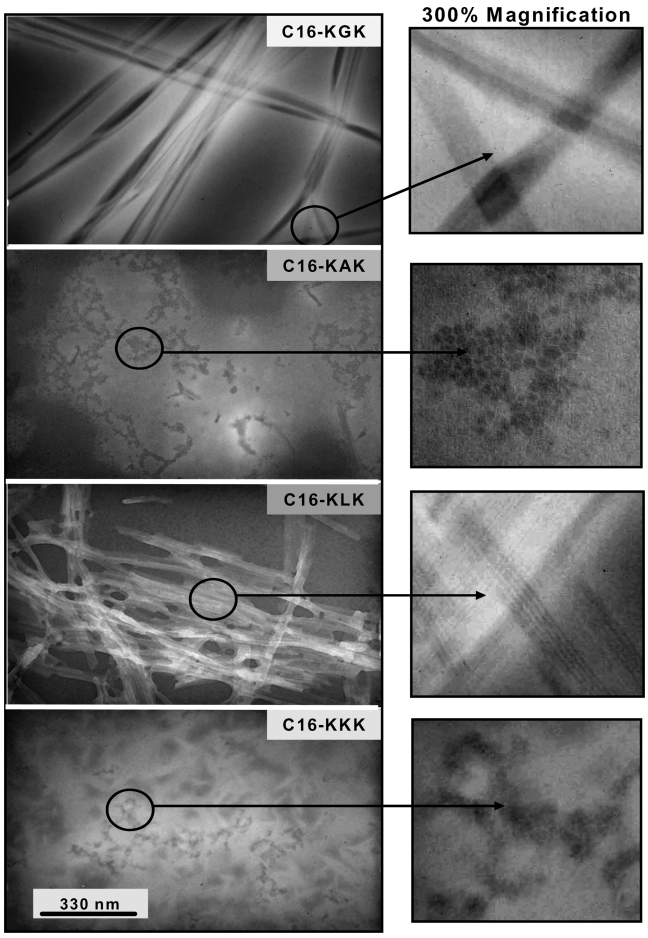

FIGURE 6: Negative staining electron microscopy of all the triamino acid lipopeptides in water and at a concentration of 200 μM.

of the peptide also does not correlate with the activity of the corresponding lipopeptides, because the order of the activity is C16-KKK > C16-KGK > C16-KLK > C16-K*K* ≫ C16-KAK, whereas the order of the hydrophobicity should be C16-KLK > C16-KAK > C16-KGK > C16-KKK> C16-K*K*. This might indicate that the organization of the lipopeptides, either in solution or when bound to a specific cell, is crucial for its biological function.

To address this possibility, we used negatively staining electron microscopy to visualize whether the lipopeptides can form large oligomers in solution. Interestingly, all of the lipopeptides could form nanostructures in solution, but with different morphologies (Figure 6). The two lipopeptides, C16-KGK and C16-KLK, formed fibrils similarly to amyloids. However, magnifications of these fibrils suggest that those formed by C16-KGK resemble nanotubes having a diameter of ~35 nm and with a single wall. In comparison, those formed by C16-KLK resemble sticks, each composed of condensed layers, each with a width of about 3 nm. Note that C16-KLK is not active against Gram-negative bacteria and also only partially disrupts its membrane. The highly dense structure of C16-KLK could prevent it from dissociating easily when bound to the outer LPS layer and then traversing into the inner membrane of Gram-negative bacteria. The idea that tight oligomerization of AMPs prevents them from traversing through the LPS layer has been demonstrated with AMPs with long peptidic chains (*24*). Compared with the active lipopeptides, C16-KAK forms well-defined micelles with a diameter of ~10 nm. The inactivity of this lipopeptide is probably related to the stability of those micelles that are not dissociated once they are bound to any type of cell. Although the most active lipopeptide C16-KKK also forms oligomers, they are too small and their size could not be visualized even at the highest magnification tested. Their small size probably allows them to interact easily with the membrane of the pathogen and to dissociate to reach and permeate the pathogen's cytoplasmic membranes. Overall, it seems that, compared to lipopeptides with longer peptidic chains and shorter fatty acid chains, the activity of the short lipopeptides is affected more by their different organizations rather than their precise structure and/or sequence.

Most natural antimicrobial lipopeptides act via two major mechanisms: (i) inhibiting the synthesis of cell wall compnents such as (1,3)-β-D-glucan or chitin (*48*−*51*) and (ii) inducing membrane lysis (*7, 8, 12, 13, 16*−*18, 52, 53*). Our data suggest that, similar to most native AMPs, a major target of these lipopeptides is the membrane of the pathogenic microorganisms. This conclusion is based on three complementary assays. (i) We monitored the ability of the lipopeptides to dissipate the membrane potential of both *E. coli* and *S. aureus*. This can happen if the peptide disrupts the integrity of the cytoplasmic membrane. However, this is not sufficient, since peptides can permeate the membrane to small molecules such as ions without killing the bacteria (*45, 54*). Indeed, the most active peptides completely dissipated the membrane potential, while the nonactive lipopeptides had only a slight effect (Figure 2). (ii) We monitored the entrance of the cationic dye SYTOX green into the microorganism (Figure 3). SYTOX green cannot enter into an intact cell unless its membrane is disrupted significantly by a membrane active compound. Once inside the cell, the florescence of the dye increases drastically owing to its binding to intracellular nucleic acids. Since the entrance of SYTOX requires significant membrane damage, only the potent lipopeptides were active in this assay. (iii) We used negatively staining electron microscopy to show that indeed only the biologically active lipopeptides created massive disruption of the cell wall and membrane of the corresponding microorganisms. Overall, we found a direct correlation between the MICs of the lipopeptides on the different microorganisms (Table 1) and their ability to disrupt and increase the permeability of the cytoplasmatic membrane of these microorganisms.

Another important finding in this study is the ability of these lipopeptides to cure fungal infection in animal models. Fungi of the genus *C. albicans* are part of the normal human flora; however, *C. albicans* can cause diseases, and it is the most frequently isolated pathogen in humans (*55, 56*). It is a ubiquitous fungal organism that often colonizes the skin and the mucosal surfaces of normal individuals and causes a variety of different superficial diseases. However, when the normal host defense mechanisms are impaired, *C. albicans* can cause serious systemic infection (*57, 58*). Here we infected mice skin with pathogenic *C. albicans* by subcutaneous injection of *C. albicans* spores complexed to Cytodex beads (*46*) and treated them by administering the lipopeptides 1 h postinfection and with one dose every day for the next 3 days. Histological examination of skin samples stained with Periodic acid−Schiff indicated that the lipoeptides significantly reduced the number of fungal spores and hyphal forms at the mice skin and inhibited the *C. albicans*

skin infection. The most efficient lipopeptide was C16-K*K*K, although C16-K*K* and C16-KGK also revealed significant *in vivo* antifungal activities.

In summary, we show that lipopeptides with very short peptidic chains are potent antimicrobial agents *in vitro* and *in vivo* against pathogenic microorganisms. The sequence of the peptidic moiety determines the extent of antimicrobial activity and cell specificity: changing one amino acid out of three controls the potency and cell selectivity of these lipopeptides. Furthermore, despite the extremely short length of the peptide chain, their plausible mode of action supports a membranolytic or detergent-like effect (*59*).

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BI8011675