pH-Dependent Antifungal Lipopeptides and Their Plausible Mode of Action†

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ABSTRACT: Antimicrobial peptides and lipopeptides play an essential protective role in the innate immune system of all organisms. Despite many studies, the factors that dictate their cell-selectivity and pH-dependent activity are yet not clear. This is important because various organs of the human body have an acidic pH environment, for example, the vagina, gastric lumen, cryogenic dental foci, and lung-lining fluids in cystic fibrosis and asthma. In this study we synthesized a new group of lipopeptides by conjugating dodecanoic acid (DDA) to the N-termini of 12-mer peptides LXXLLXXLLXXL (L_6X_6, X = Lys, His, Arg, and all the leucines are D-amino acid enantiomers) and investigated their pH-dependent biological activity and a plausible mode of action by using model phospholipids mimicking bacterial, mammalian, and fungal membranes. The data revealed that, depending on the basic amino acid incorporated, the lipopeptides are active against both bacteria and fungi or solely toward fungi. Furthermore, their activity is expressed at an acidic pH alone, neutral pH alone, or at both environments. Determination of secondary structure, membrane leakage experiments, surface plasmon resonance (SPR) binding experiments, and transmission electron microscopy suggest the involvement of a membranolytic effect. This mode of action, which should make it hard for the microorganism to develop resistance, their selective and pH-dependent activity, as well as pharmacological advantages due to the presence of D-amino acids, make them potential candidates for the treatment of mycoses in organs, under various pH environments, especially in cases where the bacterial flora should not be harmed.

Over the past few years, there has been a dramatic increase in the rate of fungal infections (*1*), for example, systemic infections appearing primarily in solid organs and in allogenic bone marrow transplantation recipients treated with immunosuppressants and patients receiving cancer chemotherapy (*2*). Mycoses can infect patients with an intact immune system as well. Because of the eukaryotic nature of fungal cells, it is difficult to find selective antifungal drugs. Currently, the antifungal pharmacopeia consists of two main families of drugs: the azoles family, which inhibits sterol formation, and polyenes, which bind to mature membrane sterols and disrupt the fungal membrane (*3*). However, the high toxicity of polyenes and the development of resistance against drugs from the azole family (*1*, *2*) has increased the demand for the development of new antifungal drugs with new modes of action.

To this end, three major families with a broad spectrum of antifungal activity were investigated. The first one contains innate immunity polycationic antimicrobial peptides (AMPs) (*4*−*7*), which serve as a nonspecific defensive mechanism by most known organisms, from prokaryotes such as bacteria to arthropods, vertebrates, and mammals including humans. AMPs complement the highly specific and relatively slow adaptive immune system. The net positive charge of AMPs facilitates interaction with anionic cell components (*8*−*12*). These include lipopolysaccharides (LPS) in Gram-negative bacteria and lipotechoic acid (LTA) in Gram-positive bacteria, as well as the anionic phospholipids in both types of bacteria. Because the membrane of fungal cells is not highly negatively charged (∼8% negatively charged phosphatidylinositol), most antimicrobial peptides are not antifungal. Furthermore, antifungal AMPs are usually also antibacterial and toxic to mammalian cells (RBCs).[1] Their mode of action is at least to partially permeate the target cell.

A second family includes lipopeptides that are produced nonribosomally in bacteria, yeast, and fungi. The peptidic moiety, which is bound to a specific acyl chain, is composed of hydrophobic and acidic L- and D-amino acids (*13*−*16*). They act via one of the following two modes of action: (i) Inhibiting the synthesis of cell wall components such as (1,3)-β-D-glucan (echinocandins) (*17*, *18*). One member of this family, caspofungin, is the first approved antifungal agent (*18*). Similarly to other drugs that act on a specific target, fungi can develop resistance to these types of lipopeptides. (ii) Direct membrane lysis resulting in irreversible damage to the fungal cell wall (*19*−*22*). Daptomycin, recently approved by FDA, acts partially via this mode of action in the presence of Ca^{2+} ions (*16*). Although this group has great

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[1] Abbreviations: ATR-FTIR, attenuated total reflectance Fourier transform infrared; CD, circular dichroism; CFU, colony-forming units; hRBC, human red blood cells; PBS, phosphate-buffered saline; PI, phosphatidylinositol; PE, phosphatidylethanolamine; RP-HPLC, reverse-phase high-performance liquid chromatography.

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potential as a therapeutic agent, their high toxicity toward mammalian cells is a major drawback (*19*, *23*).

Recently, a third family of synthetic antifungal lipopeptides was reported. It is composed of polycationic peptides covalently linked to aliphatic acids with variable lengths (*24*). Attachment of the aliphatic acids compensates for the hydrophobicity of the peptidic chain, and the resulting lipopeptides are endowed with a broad spectrum of potent antibacterial and antifungal activity without being hemolytic at their MICs (*25*). In another study, acyl analogues enhanced the antibacterial activity of a peptide fragment of human lactoferrin that originally had weak antibacterial activity (*26*).

Antifungal and antibacterial agents are studied mainly for their function at a neutral pH. However, various organs of the human body have an acidic pH environment. These include the vagina (*27*), gastric lumen, cryogenic dental foci (*28*), and lung-lining fluids in cystic fibrosis and asthma (*29*). Therefore, the development of pH-dependent antimicrobial and antifungal agents is an important need. In line with this, limited studies were reported on antimicrobial peptides that are active mainly against bacteria in both neutral and acidic pH environments (*9*, *30*, *31*). However, since in several cases, such as vaginal candidosis, bacteria flora has a crucial role in controlling the population of candida (*27*), selective antifungal agents are needed.

Here, we synthesized a group of lipopeptides by conjugating dodecanoic acid to the N-termini of a series of peptides having the sequence LXXLLXXLLXXL (X = Lys, His, Arg, and all the leucines are D-amino acid enantiomers). The lipopeptides were examined for their pH-dependent biological activity, as well as their mode of action at acidic and physiological milieus by using various biophysical methods. The lipopeptides expressed different target specificities at different pHs, depending on the type of the positively charged amino acids incorporated. Our results are discussed regarding the unique composition and biological function of the lipopeptides that, together with their membranolytic mode of action, make them potential candidates for the treatment of mycoses in organs, under various pH environments, without harming the native bacterial flora.

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-(1H-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). 8-Anilinonaphthalene 1-sulfonate (ANS) was purchased from Sigma. 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. Briefly, after removal of the Fmoc from the N-terminus of the peptide with a solution of 20% piperidine in DMF, the fatty acid (7 equiv, 1 M in DMF) was coupled to the resin under similar conditions used for the coupling of an amino acid. The peptides were cleaved from the resin with 95% trifluoroacetic acid (TFA) and were purified by RP-HPLC on a C18 Bio-Rad semipreparative column (250 mm × 10 mm, 300 Å pore size, 5 μm particle size). The purified peptides were shown to be homogeneous (>98%) by analytical RP-HPLC. The elution time of the lipopeptides increased by ~10 min, indicating an increase in hydrophobicity due to the attachment of the fatty acid. Electrospray mass spectroscopy was used to confirm their molecular weight, and amino acid analysis was used to confirm the composition of the peptidic moiety.

Antifungal Activity. The antifungal activity of the peptides and their fatty acid-conjugated analogues was measured according to 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 or 5.5, 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) or 48−72 h for *Candida albicans* (ATCC 10231), using a Binder KB115 incubator. Growth inhibition was determined by measuring the absorbance at 620 nm in a microplate autoreader El309 (Bio-tek Instruments). Antifungal activity is expressed as the minimal inhibitory concentration (MIC), the concentration at which no growth was observed.

Antibacterial Activity. The antibacterial activity of the peptides 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 (LB medium adjusted to pH 7.4 or 5.5) 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 (adjusted to pH 7.4 or 5.5, respectively). Inhibition of growth was determined by measuring the absorbance at 492 nm with a Microplate autoreader El309 (Bio-tek 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 *Escherichia coli* ATCC 25922, *Escherichia coli* ATCC D21 *Pseudomonas aeruginosa* ATCC 27853, *Acinetobacter baumannii* ATCC 19606, *Enterococcus feaecalis ATCC 25922*, *Staphylococcus aureus* ATCC 6538P, and *Enterbacter cloacae* ATCC 49141.

Hemolysis of Human Red Blood Cells (hRBCs). Fresh hRBCs with EDTA were rinsed three times with PBS (35 mM phosphate buffer/0.15 M NaCl, pH 7.3) by centrifugation for 10 min at 800*g* and resuspended in PBS. Lipopeptides dissolved in PBS were then added to 50 μL of a solution of the stock hRBCs in PBS to reach a final volume of 100 μL (final erythrocyte concentration, 4% v/v). The resulting suspension was incubated with agitation for 60 min at 37 °C. The samples were then centrifuged at 800*g* for 10 min. The 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 Triton 1%, respectively.

Preparation of Liposomes. Small unilamellar vesicles (SUVs) were prepared by sonication. Briefly, dry lipids were dissolved in chloroform/MeOH (2:1, v/v). The solvents were then evaporated under a stream of nitrogen and then lyophilized overnight. The lipids were resuspended in the appropriate buffer (10 mg/mL) with vortexing, and the resulting lipid dispersions were sonicated (10−30 min) in a bath-type sonicator (G1125SP1 sonicator; Laboratory Supplies Company, Inc., Hicksville, NY) until the turbidity had cleared. The vesicles were visualized using a JEOL JEM 100B electron microscope (Japan Electron Optics Laboratory Co., Tokyo, Japan). Lipid films were prepared from three types of phospholipids, PC/PE/PI/ergosterol (5:4:1:2 w/w/w/w), PE/PG (7:3 w/w), and PC/cholesterol (10:1 w/w), which mimic the outer leaflets of the plasma membranes of *C. albicans* (*32*), *E. coli* (*33*), and *hRBC* (*34*), respectively.

Membrane Permeability Studies. Membrane permeation was assessed utilizing the calcein release assay (*35*). Briefly, calcein (60 mM, self-quenching concentration) was entrapped in SUVs composed of PC/cholesterol (10:1, w/w) or PE/PG (7:3, w/w) or PE/PC/PI/ergosterol (5:4:1:2) 10 mg/mL. The buffer was 10 mM Hepes and 150 mM NaCl (pH 7.4). The nonencapsulated calcein was removed from the liposome suspension by gel filtration, using a Sephadex G-50 (Pharmacia) column connected to a low-pressure LC system (Pharmacia). The eluent was monitored by UV absorbance (λ = 280 nm), and the vesicles' peak was collected and diluted 10-fold in the same buffer. A final concentration of 2.5 μM SUVs dissolved in the same buffer adjusted to pH 5.5 or 7.4 was used. The calcein dye trapped inside the vesicles is self-quenched; membrane permeation was detected by an increase in fluorescence as a consequence of adding lipopeptide-induced membrane permeability and calcein leakage. The lipopeptides were added in an increasing lipopeptide-to-lipid ratio (0.007−0.8 lipopeptide/lipid). The fluorescence was monitored at room temperature (λ_{ex} = 485 nm, λ_{em} = 515 nm). Complete dye release (used as 100% activity) was obtained after the vesicles were disrupted with Triton X-100 (final concentration of 0.1%). Under the experimental conditions, in the absence of peptide, the leakage rate was less than 1% in 5 h.

Circular Dichroism (CD) Spectroscopy. The CD spectra of the peptides were measured in an Aviv 202 spectropolarimeter. The spectra were scanned with a thermostated quartz optical cell with a path length of 1 mm. Each spectrum was recorded at 1 nm/s intervals with an average time of 20 s, at a wavelength range of 195−260 nm. The peptides were scanned at a 100 μM concentration in PBS alone and in the presence of 1% LPC micelles dissolved in 35 mM phosphate buffer adjusted to pH 7.4 or 5.5.

Binding Analysis by Surface Plasmon Resonance (SPR). SPR experiments were carried out with a BIAcore 3000 analytical system (Biacore, Uppsala, Sweden) using L1 sensor chips (BIAcore). The L1 sensor chip surface consists of a thin dextran matrix modified with lipophilic compounds on a gold surface, where a lipid bilayer is prepared by the capture of SUVs (*36*). We performed a protocol as previously described by Mozsolits et al. (*36*) and Papo et al. (*37*, *38*). The running buffer used for all experiments was PBS without calcium and magnesium (adjusted to pH 7.4 or 5.5). The washing solution was 40 mM N-octyl β-D-glucopyranoside. All solutions were freshly prepared, degassed, and filtered through 0.22-μm pores. The operating temperature was 25 °C. The L1 chip was then installed, and the dextran surface was cleaned by two injections of N-octyl β-D-glucopyranoside (25 μL, 40mM), at a flow rate of 5 μL/min. PC/Chol (10:1 w/w), PC/PE/PI/ergosterol (5:4:1:2 w/w/w/w), or PE/PG (7:3 w/w) SUVs solution (80 μL PBS, 0.5 mM) was then applied twice to the chip surface at a flow rate of 2 μL/min. To remove any multilamellar structures from the lipid surface, we increased the flow rate to 50 μL/min, which resulted in a stable baseline corresponding to the lipid bilayer linked to the chip surface. The bilayer linked to the chip surface was then used to study the peptide−membrane binding. Peptide solutions (100 μL of 0.39−12.5 μM peptide in PBS) were injected onto the lipid surface at a flow rate of 5 μL/min. PBS alone then replaced the peptide solution for 1000 s to allow for peptide dissociation. SPR detects changes in the reflective index of the surface layer of peptides and lipids in contact with the sensor chip. A sensogram is obtained by plotting the SPR angle against time. This change in the angle is then translated to response units. Analysis of the peptide−lipid binding event was performed from a series of sensograms collected at different peptide concentrations. The peptide-to-lipid molar ratio was calculated by using a channel volume of 0.02 μL and the following ratio between RU and lipid loading: 5000 RU corresponds to an increase in chip loading of 6×10^{-12} mol lipids.

Determination of Lipopeptides Aggregation by Using 8-Anilinonaphthalene 1- Sulfonate (ANS). ANS partitions into hydrophobic environments concomitant with an increase in its fluorescence intensity and, therefore, has been used to determine the oligomerization of polypeptides or the micellar concentration of detergents in solutions (*39*−*41*). In a typical experiment, a stock solution of ANS in methanol (0.01M) was diluted into PBS (final concentration of 10^{-5} M) adjusted to pH 7.4 or 5.5. The lipopeptides were then added in increased concentrations (0.78−12.5 μM), and the fluorescence was recorded at room temperature with excitation set at 350 nm and emission set at 530 nm.

Electron Microscopy. Samples containing *C. albicans* ATCC 10231 (3.5×10^7 CFU/mL) were incubated with or without (control) the lipopeptides dissolved in PBS (adjusted to pH 7.4 and 5.5) at their MIC for 15 min. The fungi were fixed by incubation with 1% glutaraldehyde in PBS for 20 min. A drop containing the fungi was deposited onto a carbon-coated grid and negatively stained with 1% uranil acetate. The grids were examined on a JEOL JEM 100B electron microscope (Japan Electron Optics Laboratory Co., Tokyo, Japan).

Table 1: Peptides' Designations, Sequences, and RP-HPLC Retention Times

peptide designation	sequence[a]	RP-HPLC retention time[b] (min)
DDA-DL_6K_6	DDA-LKKLLKKLLKKL	24.3
DDA-DL_6H_6	DDA-LHHLLHHLLHHL	28
DDA-DL_6R_6	DDA-LRRLLRRLLRRL	25.7
DDA- $DL_6K_3H_3$	DDA-LKHLLKHLLKHL	26
DDA-$DL_6R_3H_3$	DDA-LRHLLRHLLRHL	27

[a] Underlined amino acids are D-enantiomers. All the peptides are amidated at their C-terminus. [b] HPLC gradient 10−90% acetonitrile in water during 40 min both containing 0.5% TFA.

Table 2: MICs of the Peptides (μM) against Yeasts and Fungi at pH 7.4

peptide designation	*A. flavus* (ATCC 9643)	*A. fumigatus* (ATCC26430)	*C. albicans* (ATCC10231)
DDA-DL_6K_6	0.78	12.5	3.1
DDA-DL_6H_6	>100	>100	>100
DDA-DL_6R_6	0.78	3.1	3.1
DDA- $DL_6K_3H_3$	9.4	25	12.5
DDA-$DL_6R_3H_3$	9.4	25	12.5
Amphotericin B	3.1	2.3	3.1

RESULTS

Peptide Design and Sequences. We synthesized five diastereomeric peptides, each composed of 12 amino acids with the sequence LXXLLXXLLXXL (X = Lys, His, Arg) and amidated at their C-termini. All the Leu are D-amino acid. Previous studies have suggested that the parental diastereomers should be devoid of antimicrobial and hemolytic activities due to their low hydrophobicity (*42*) and that an α-helical structure is required when the conjugated fatty acid is shorter than myristic acid (*24*). The peptides were designed to create an amphipathic α-helical structure in their all L-form, based on Schiffer and Edmondson's wheel projection (*43*) (figure not shown), and Lauric acid (dodecanoic acid) was attached to their N-termini. Table 1 shows the sequences of the lipopeptides, their designations, molecular weights, and RP-HPLC retention times. Since all the parental peptides lacking the fatty acid were inactive in the biological and model membrane assays, only the lipopeptides are shown.

Antifungal Activity of the Lipopeptides. Antifungal activity was assayed against representative pathogenic fungi that are common in human fungal infections. The culture medium for antifungal assays was adjusted to two different pH values, neutral pH 7.4 and acidic pH 5.5. The antifungal drug Amphotericin B served as a control and gave the reported MIC values. MIC values at pH 7.4 are presented in Table 2, and at pH 5.5 in Table 3. The results show a pH-dependent activity. DDA-DL_6H_6 was practically inactive at pH 7.4. However, at pH 5.5, this lipopeptide was highly active against all fungal species tested. In comparison, DDA-DL_6K_6 was very potent at pH 7.4 but lost its antifungal activity at pH 5.5. The other two lipopeptides containing only three Histidines preserved activity at both pH values.

Antibacterial Activity of the Lipopeptides. The activities of the lipopeptides against four species of Gram-negative bacteria and three species of Gram-positive bacteria are shown in Tables 4 and 5 for pH 7.4 and 5.5, respectively.

Table 3: MICs of the Peptides (μM) against Yeasts and Fungi at pH 5.5

peptide designation	*A. flavus* (ATCC 9643)	*A. fumigatus* (ATCC26430)	*C. albicans* (ATCC10231)
DDA-DL_6K_6	>100	75	100
DDA-DL_6H_6	25	6.2	9.4
DDA-DL_6R_6	50	6.2	12.5
DDA- $DL_6K_3H_3$	50	12.5	12.5
DDA-$DL_6R_3H_3$	50	6.2	25
Amphotericin B	6.2	3.1	9.4

The antibiotic Gentamicin served as a control. Only the Lys-containing peptides expressed antimicrobial activity, although marginal in most cases, whereas all other lipopeptides were practically devoid of antimicrobial activity.

Hemolytic Activity of the Lipopeptides. The hemolytic activity of the lipopeptides against the highly diluted solution of human erythrocytes is shown in Figure 1. All the lipopeptides are practically nonhemolytic at their MICs against fungi. However, the Arg-containing peptides expressed significant hemolytic activity at higher concentrations.

Mode of Action Studies. The lipopeptides, which contained both H and either K or R, that is, DDA-$DL_6K_3H_3$ and DDA-$DL_6R_3H_3$, preserved the activities of the two corresponding parental peptides containing only one of the basic amino acids. Therefore, detailed mode of action studies were further conducted only with DDA-DL_6K_6, DDA-DL_6H_6, and DDA-DL_6R_6.

Membrane Disruption Induced by the Lipopeptides. The lipopeptides were investigated for their ability to induce calcein release from SUVs composed of three types of phospholipids mimicking the membrane composition of fungi, bacteria, and mammalian cells. Lipopeptides, at increasing concentrations, were added to a suspension of vesicles encapsulated with calcein (2.4 μM phospholipids) at pH 7.4 or 5.5, and membrane permeability was followed by monitoring the recovery of fluorescence. The level of maximum leakage reached as a function of the peptide-to-lipid molar ratio is shown in Figure 2 parts A, B, and C for PC/PE/PI/ergosterol (5:4:1:2, w/w/w/w), PE/PG (7:3, w/w), and PC/cholesterol (10:1, w/w), respectively. DDA-DL_6K_6 and DDA-DL_6R_6 permeated all types of vesicles at pH 7.4, whereas DDA-DL_6H_6 was inactive. However, at pH 5.5, DDA-DL_6H_6 was also highly active, sometimes more than the other two lipopeptides. Interestingly, despite the high net positive charge of the lipopeptides, they were active similarly at pH 7.4 on phospholipids mimicking mammalian and fungal membranes but had lower activity on bacterial membranes.

Secondary Structures of the Lipopeptides in Different Solutions, As Determined by Circular Dichroism Spectroscopy (CD). The secondary structure of the lipopeptides (100 μM) was determined in PBS, 40% TFE/water, 1% SDS, and 1% LPC. We could not measure the CD spectra in vesicles due to light scattering. The lipopeptides did not show significant signal in PBS, in 40% TFA/water (data not shown), and SDS (Figure 3). However, all of them had a signal characteristic of a partial α-helical structure in 1% LPC, and for each lipopeptide, the structure was independent of the pH (Figure 3). The parental peptides had no structure with or without 1% LPS at both pH values (data not shown).

Table 4: MICs of the Peptides (μM) against Bacteria at pH 7.4

	Gram-negative bacteria				Gram-positive bacteria		
peptide designation	*E. coli* (ATCC 25922)	*E. coli* D21	*P. aeruginosa* (ATCC 27853)	*A. baumannii* (ATCC 19606)	*S. aureus II* (ATCC 6538P)	*E. feaecalis* (ATCC 19433)	*E. cloacae* (ATCC 49141)
DDA-DL_6K_6	75	25	25	25	25	50	6.2
DDA-DL_6H_6	>100	>100	>100	100	100	100	>100
DDA-DL_6R_6	>100	50	50	>100	31	50	19
DDA-$DL_6K_3H_3$	100	50	50	50	22	25	100
DDA-$DL_6R_3H_3$	100	100	100	100	100	25	62

Table 5: MICs of the Peptides against Bacteria (μM) at pH 5.5

	Gram-negative bacteria				Gram-positive bacteria		
peptide designation	*E. coli* (ATCC 25922)	*E. coli* D21	*P. aeruginosa* (ATCC 27853)	*A. baumannii* (ATCC 19606)	*S. aureus II* (ATCC 6538P)	*E. feaecalis* (ATCC 19433)	E. cloacae (ATCC 49141)
DDA-DL_6K_6	50	25	12.5	25	>100	50	6.2
DDA-DL_6H_6	>100	100	100	>100	>100	>100	>100
DDA- DL_6R_6	100	50	50	75	50	>100	>100
DDA-$DL_6K_3H_3$	100	50	50	50	25	25	100
DDA-$DL_6R_3H_3$	100	100	100	>100	100	100	100

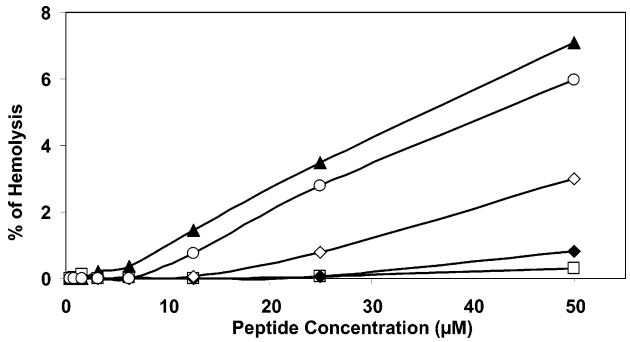

FIGURE 1: Dose response of the hemolytic activity of the lipopeptides toward 4% hRBC. Designations are DDA-DL_6K_6 (□), DDA-DL_6H_6 (♦), DDA-DL_6R_6 (▲), DDA-$DL_6K_3H_3$ (◊), and DDA-$DL_6R_3H_3$ (○).

Note that all the spectra in LPC have positive values of elipticity due to the incorporation of the D-amino acids.

Binding of the Lipopeptides to Lipid Bilayers Monitored by Surface Plasmon Resonance (SPR). PE/PG (7:3, w/w), PC/PE/PI/ergosterol (5:4:1:2, w/w), and PC/cholesterol (10:1, w/w) bilayers were adsorbed onto L1 sensor chips. Typical sensograms of the lipopeptides (at 6.25 and 12.5 μM) binding to PC/PE/PI/ergosterol (5:4:1:2, w/w) membranes at neutral and acidic pHs are shown in Figure 4. Since the shapes of the sensograms for the other two types of phospholipids were similar, they are not shown, but the maximal responses obtained at 12.5 μM for all of them are shown in Table 6. The RU signal intensity, which corresponds to the amount of bound lipopeptide, increased as a function of the lipopeptide's concentration. The finding that some of the lipopeptides were only partially released from the membrane, even after overnight flow of buffer, did not allow us to calculate binding constants. However, the figures clearly show that, compared with DDA-DL_6K_6 and DDA-DL_6R_6, which bind strongly at both pHs, DDA-DL_6H_6 binds only at an acidic pH. Interestingly, in contrast with the differences in the biological function of the lipopeptides toward the various target cells, a similar trend of binding was observed independent of the type of lipids used. For example, DDA-DL_6H_6 binds similarly to all types of lipids at an acidic pH, but it is inactive on bacteria under these conditions. Note also that the binding of DDA-DL_6H_6 at an acidic pH increased markedly when applied at concentrations higher than 6.25 μM. The parental peptides lacking the dodecanoic acid had very weak binding to all of the different membranes at both pH values (results not shown).

The Aggregation of the Lipopeptides Determined by Using ANS. The lipopetides at increasing concentrations were added to an ANS solution (10^{-5} M), and the fluorescence was recorded. The dose-response of the fluorescence as a function of the concentration of the lipopeptides is shown in Figure 5, panels A and B, for pHs 7.4 and 5.5, respectively. The parental peptides were used as controls, and no increases in fluorescence were observed (data not shown). The data reveal a significantly greater maximal change in the fluorescence in the presence of DDA-DL_6R_6 and DDA-DL_6H_6 compared with DDA-DL_6K_6 at pH 7.4 and with DDA-DL_6R_6 at pH 5.5. Although the extent of aggregation at the different pHs cannot be determined exclusively by this assay, the data suggest that DDA-DL_6K_6 is the least hydrophobic peptide and probably the least aggregated one, under the experimental conditions at both pHs.

Visualization of Candida albicans by Using Electron Microscopy. C. albicans was treated with DDA-DL_6K_6 and DDA-DL_6R_6 at pH 7.4 and with DDA-DL_6H_6 at pH 7.4 and 5.5 for 15 min at their MIC values and visualized by using transmission electron microscopy (Figure 6). DDA-DL_6K_6 and DDA-DL_6R_6 at pH 7.4 caused a massive disruption of a large portion of the cell wall of *C. albicans* at pH 7.4 (Figure 6B) compared with DDA-DL_6H_6, which was inactive (Figure 6C). However, at pH 5.5, DDA-DL_6H_6 was active similarly to DDA-DL_6K_6 (Figure 6D).

DISCUSSIONS

An important outcome of this study is a new group of pH-dependent antifungal peptides composed of membrane-inactive cationic peptides covalently linked to dodecanoic acid. All parental peptides are derived from a similar peptidic backbone but differ in the type of the basic amino acid. Compared with Lys and Arg, which are protonated at pH 7.4, His has a pK_a of approximately 6.5. Consequently, His-rich lipopeptides have a net positive charge below ~pH 6.5

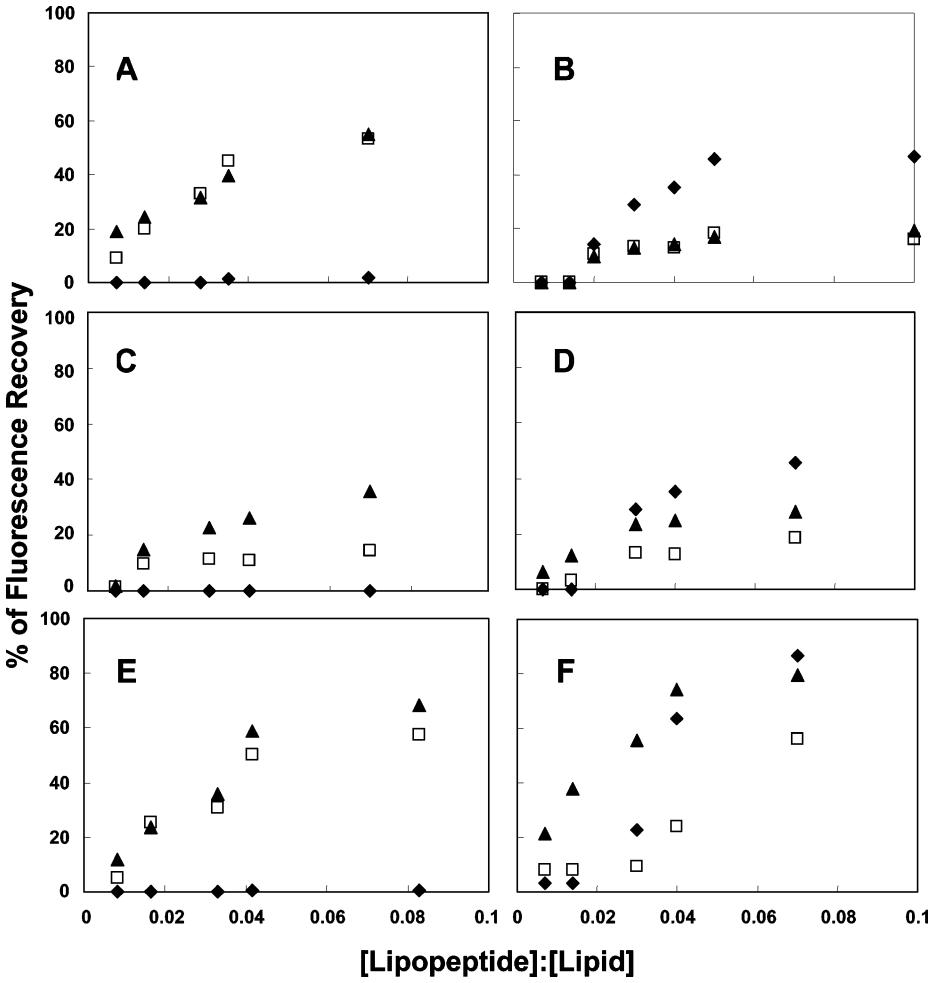

FIGURE 2: Calcein release induced by the lipopeptides. Lipopeptides were added to SUVs composed of different phospholipids (2.5 μM final concentration) and encapsulated with calcein. (A) PC/PE/PI/ergosterol (5:4:1:2) pH 7.4; (B) PC/PE/PI/ergosterol (5:4:1:2) pH 5.5; (C) PE/PG (1:1) pH 7.4; (D) PE/PG (1:1) pH 5.5; (E) PC/Chol (10:1) pH 7.4; and (F) PC/Chol (10:1) pH 5.5. Lipopeptides designations are DDA-DL_6H_6 (♦), DDA-DL_6K_6 (□), and DDA-DL_6R_6 (▲).

but are predominantly uncharged at pH 7.4. Therefore, if the charge is crucial for their activities of such peptides, then they are expected to be active only at acidic pH values. Here, we show that the type of the basic amino acid can endow different activities to the resulting lipopeptides (Tables 2−5). The most interesting lipopeptide is DDA-DL_6H_6. It lacks activity at a neutral pH but acquires potent antifungal activity at an acidic pH. Moreover, the peptide lacks antibacterial activity. This is very important in organs in which the native flora is required for protection, such as in the case of vaginal candidosis.

Interestingly, although DDA-DL_6K_6 and DDA-DL_6R_6 should have similar pH-dependent activities because they are protonated at both pHs, DDA-DL_6K_6 is antifungal at a neutral pH but not at an acidic pH, whereas DDA-DL_6R_6 is active at both pHs (Tables 2 and 3). Furthermore, DDA-DL_6K_6 is the only peptide endowed with antibacterial activity at both pHs. These data can be partially explained by taking into account the reduced hydrophobicity and probably also oligomeric state of DDA-DL_6K_6 compared with the other two lipopeptides (Figure 5). Peptides with increased hydrophobicity and/or oligomeric state could bind to LPS, a component of the outer membrane of Gram-negative bacteria (*44*) or techoic acid in the cell wall of Gram-positive bacteria, which makes it difficult for them to traverse the bacterial cell wall (*45*, *46*). Note that substituting of only three Lys with three His made the analogue DDA-$DL_6K_3H_3$ active also at an acidic pH, whereas a similar substitution in the Arg peptide preserved activity at both pHs. Importantly, all the lipopeptides were practically not hemolytic at their MICs against a highly diluted solution of erythrocytes (Figure 1), and only the Arg-containing lipopeptides showed a marked increase in hemolytic activity after a threshold concentration had been reached.

Mode of Action. Native lipopeptides were shown to act via two different mechanisms: (i) inhibition of enzymes such as (1,3)-β-D-glucansyntase and chitin synthase (*13*, *18*, *47*) or (ii) membrane lysis (*19*, *48*, *49*). Here, mode of action studies were conducted at neutral and acidic pHs with model phospholipid membranes mimicking those of fungal, bacterial, and mammalian cells. Overall, the direct correlation between the peptide's antifungal activity (with a few exceptions), the membrane binding observed by using SPR, the ability to induce the release of calcein from vesicles which model fungal membranes, as well as the damage to the *candida* membrane visualized by EM, suggest a membranolytic mode of action.

The data obtained from the calcein leakage assay (Figure 2) can be summarized as follows: (i) DDA-DL_6H_6 is practically inactive at pH 7.4 but is the most active peptide

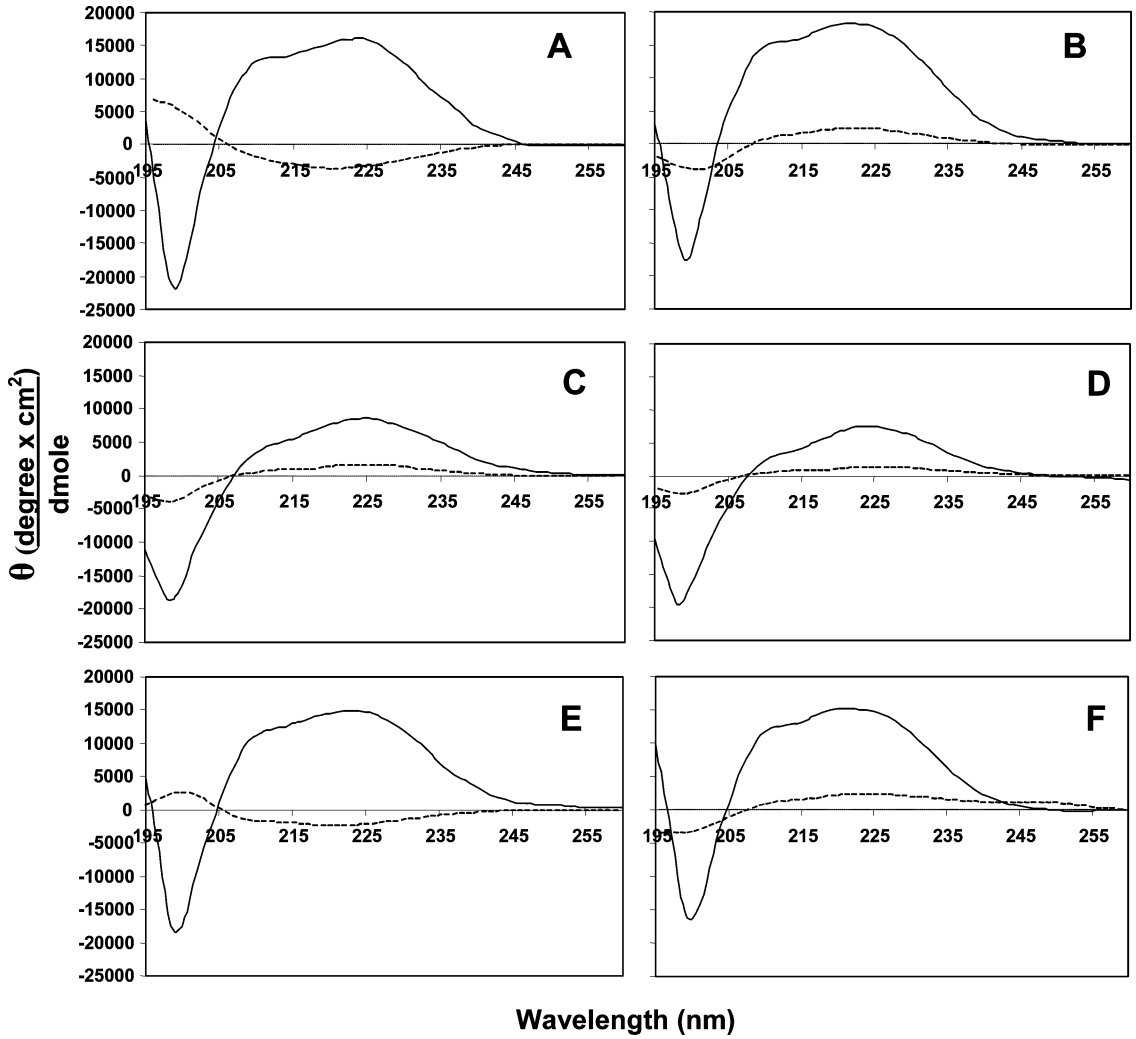

FIGURE 3: CD spectra of the lipopeptides dissolved in LPC (continuous line) and SDS (dashed line) micelles at pH 7.4 and 5.5. Spectra were taken at a peptide concentration of 100 μM. (A) DDA-DL_6H_6 pH 7.4; (B) DDA-DL_6H_6 pH 5.5; (C) DDA-DL_6K_6 pH 7.4; (D) DDA-DL_6K_6 pH 5.5; (E) DDA-DL_6R_6 pH 7.4; and (F) DDA-DL_6R_6 pH 5.5.

at pH 5.5 toward all types of vesicles. (ii) Both DDA-DL_6K_6 and DDA-DL_6R_6 are active on the three types of vesicles at both pHs. However, there are differences between their biological activities. Previous studies have shown that the ability of antimicrobial peptides to kill microorganisms does not always correlate with their ability to permeate membranes that mimic those of the corresponding microorganism (*50*). This is because they need to traverse the outer cell wall of bacteria before reaching the inner phospholipids membrane, as discussed above.

CD spectroscopy revealed that the peptides do not have any detectable secondary structure in solution and in 40% TFE/water even at high concentrations (100 μM). However, some of the lipopeptides form oligomers in solution as suggested by the ANS assay (Figure 5), most probably with the hydrophobic environment formed by the oligomerized aliphatic chains. This is because one would expect to detect a secondary structure if oligomerization is induced by the peptidic moiety (*51*). Interestingly, the peptidic chain in all of the lipopeptides formed a partial α-helix in a zwitterionic hydrophobic environment (1% LPC micelles) but not in an acidic membrane-mimetic environment (Figure 3). The CD data also show that there are no significant differences between the secondary structures of all the lipopeptides at the two pHs in a membrane-mimetic environment, indicating that the pH dependent activities are not due to differences between secondary structures. Note that circular dichroism in proteins is a phenomenon that results when chromophores (mainly nonbonding electrons of the carbonyl oxygens) in an asymmetrical environment interact with polarized light. A diastereomer, as opposed to all-L- or all-D-amino acid peptides, is composed of both D- and L-amino acids that have opposite magnetic dipole moment and optical activity. The opposite circularly polarized absorption components of the D- and L-amino acid can decrease or even eliminate the overall absorbance of the right-handed helix. Thus, the analyzed results collected from the CD, regarding the diastereomers, should not be considered quantitatively but rather qualitatively, as reflecting a partial helical secondary structure.

A second pronounced effect that could explain partially the differences in the activities of all the lipopeptides is their ability to bind phospholipid membranes as revealed by SPR (Figure 4). Although the results could not be fitted to any simple model due to the irreversible binding in several cases, they can be summarized as follows: (i) DDA-DL_6H_6 binds very weakly to all types of membranes at pH 7.4 but the binding increases dramatically (15- to 30-fold) at pH 5.5

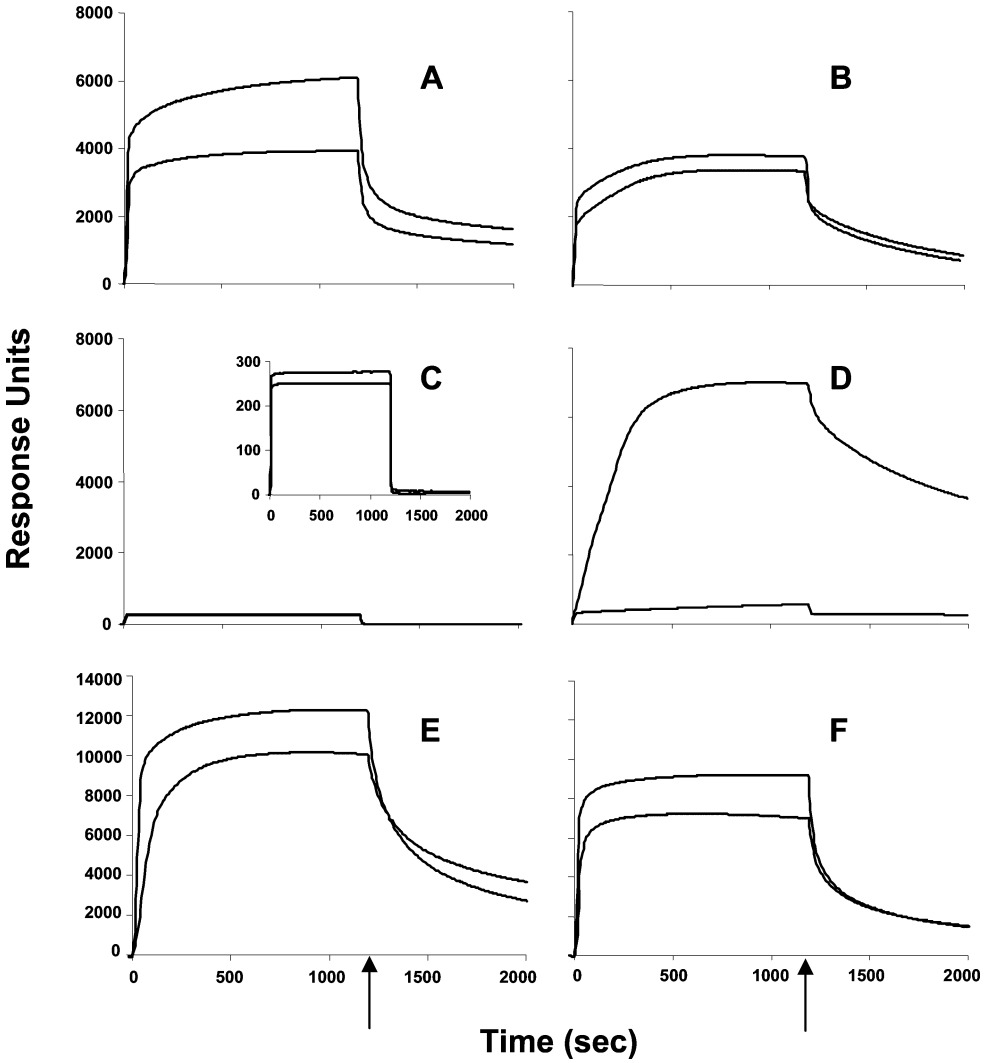

FIGURE 4: Sensograms obtained from the binding between the lipopeptides and PE/PC/PI/ergosterol membrane at pH 7.4 or 5.5. Lipopeptides' concentrations are 6.25 μM (lower curve) and 12.5 μM (upper curve). Arrows indicate the time at which the flow containing lipopeptides was replaced with flow of blank buffer. (A) DDA-DL_6K_6 pH 7.4; (B) DDA-DL_6K_6 pH 5.5; (C) DDA-DL_6H_6 pH 7.4; (D) DDA-DL_6H_6 pH 5.5; (E) DDA-DL_6R_6 pH 7.4; and (F) DDA-DL_6R_6 pH 5.5.

Table 6: Maximal Response Units of the Lipopeptides at Concentration of 12.5 μM toward Model Membranes at pHs 7.4 and 5.5

	pH 7.4			pH 5.5		
lipopeptide	PE/PG	PE/PC/PI/ergo	PC/Chol	PE/PG	PE/PC/PI/ergo	PC/Chol
DDA-DL_6K_6	5910	6030	3180	5710	3780	3760
DDA-DL_6H_6	460	277	184	2380	7000	3040
DDA-DL_6R_6	4460	12300	5130	1760	9190	7155

toward all membranes, although one would expect increased binding only toward the negatively charged membranes. (ii) There is a dramatic increase in the binding of DDA-DL_6H_6 to all the membranes at pH 5.5 after a threshold concentration of 6.25 μM has been reached. This concentration is equivalent to a lipopeptide/phospholipids molar ratio of ~0.02, at which this lipopeptide becomes active in the calcein release assay. (iii) Lipopeptides with potent antifungal activity compared with antibacterial activity showed increased binding capacity to fungal PE/PC/PI/ergo membranes compared with PE/PG membranes (Table 6). (iv) In all cases, peptides that bind phospholipids are able to induce significant calcein release from the corresponding phospholipids vesicles.

Interestingly, despite the fact that both Lys- and Arg-containing lipopeptides bind and significantly permeate zwitterionic membranes, only the Arg lipopeptide is hemolytic. This can be partially explained by the higher hydrophobicity and possibly by the oligomeric state of the Arg lipopeptide compared with the Lys lipopeptide. Previous studies have shown that increasing the hydrophobicity of antimicrobial peptides enhances their hemolytic activity (reviewed in refs *50*, *52*). The low hemolytic activity of the His lipopeptide at pH 7.4 can be explained partially to its very weak binding to PC/chol membranes as well as the lack of a net positive charge which has been shown to be important for hemolytic activity.

Finally, the EM studies also support a membranolytic mode of action (Figure 6). Incubation of *C. albicans* at pH 7.4 with the lipopeptides DDA-DL_6K_6 and DDA-DL_6R_6 at their MIC concentration for 15 min revealed extensive

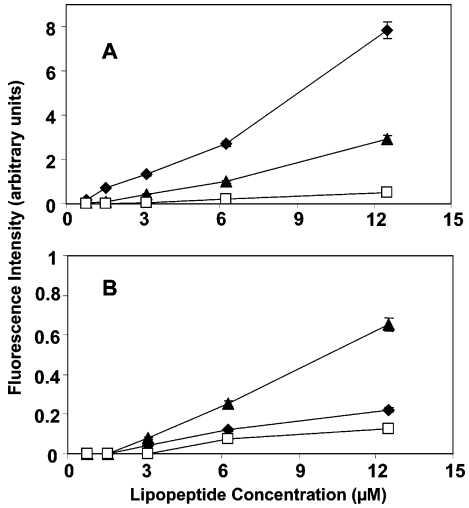

FIGURE 5: Fluorescence intensity of ANS as a function of the concentration of the lipopeptides. (A) pH 7.4; (B) pH 5.5. Lipopeptides designations are DDA-DL_6H_6 (♦), DDA-DL_6K_6 (□), and DDA-DL_6R_6 (▲).

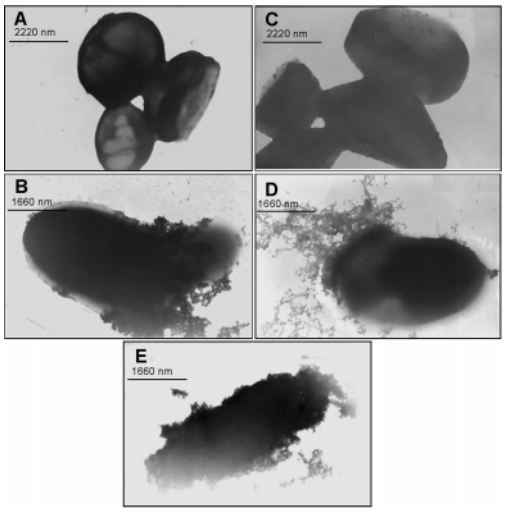

FIGURE 6: Electron micrographs of negatively stained *C. albicans* untreated and treated with the lipopeptides at neutral and acidic pH values. Peptides concentration used are their MICs, besides DDA-DL_6H_6 in which the MIC at pH 7.4 was used at both pHs. (A) Control *Candida* at pH 7.4 (a similar figure was obtained at pH 5.5 and therefore not shown); (B) treated with 3 μM DDA-DL_6K_6 at pH 7.4; (C) treated with 10 μM DDA-DL_6H_6 at pH 7.4; (D) treated with 10 μM DDA-DL_6H_6 at pH 5.5.; (E) treated with 10 μM DDA-DL_6R_6 at pH 7.5.

damage to the cell wall, as well as the loss of cytoplasmatic fluid. In addition, and in agreement with its antifungal activity, DDA-DL_6H_6 could induce similar damage only at an acidic pH.

In summary, this study demonstrates an important but not sufficient role for the positive charge of the lipopeptides in controlling cell-specific activity at different pHs. In addition, the data support a membranolytic mode of action for these lipopeptides, which should make it hard for the fungi to develop resistance. Together with their selective and pH-dependent activity, as well as the advantages due to the presence of D-amino acids, these lipopeptides are potential templates for the design of antifungal agents for the treatment of mycoses in organs at various pH values, and especially in organs with an acidic environment (e.g., vaginal candidasis), without damaging the natural bacterial flora.

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