A New Group of Antifungal and Antibacterial Lipopeptides Derived from Non-Membrane Active Peptides Conjugated to Palmitic Acid

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Running title: Palmitoylated antimicrobial lipopeptides

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Abbreviations used: 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.

Key words: Antimicrobial peptides; lytic peptides; peptide-membrane interaction
SUMMARY

We report on the synthesis, biological function, and a plausible mode of action of a new group of lipopeptides with potent antifungal and antibacterial activities. These lipopeptides are derived from positively charged peptides containing D- and L-amino acids (diastereomers) which are palmitoylated (PA) at their N-terminus. The peptides investigated have the sequence K₄X₇W, where X designates one of the following amino acids: Gly, Ala, Val, or Leu (designated D-X peptides). The data revealed that PA-D-G and PA-D-A gained potent antibacterial and antifungal activity, despite the fact that both parental peptides were completely devoid of any activity toward microorganisms and model phospholipid membranes. In contrast, PA-D-L lost the potent antibacterial activity of the parental peptide but gained and preserved partial antifungal activity. Interestingly, both D-V and its palmitoylated analog were inactive toward bacteria, and only the palmitoylated peptide was highly potent toward yeast. Both PA-D-L and PA-D-V lipopeptides were also endowed with hemolytic activity. Mode of action studies were performed by using tryptophan fluorescence, ATR-FTIR and CD spectroscopy, as well as transmembrane depolarization assays with bacteria and fungi. The data suggest that the lipopeptides act by increasing the permeability of the cell membrane, and that differences in their potency and target specificity are the result of differences in their oligomeric state and ability to dissociate and insert into the cytoplasmic membrane. These results provide insight regarding a new approach of modulating hydrophobicity and the self-assembly of non-membrane interacting peptides, in order to endow them with both antibacterial and antifungal activities urgently needed to combat bacterial and fungal infections.
INTRODUCTION

Together with the growing number of individuals with impaired host defenses, invasive mycoses have emerged as major causes of morbidity and mortality in the last decade (1-5). Although the spectrum of fungal pathogens has changed, the vast majority of invasive fungal infections are still due to Aspergillus and Candida species (4-6). Owing to their eukaryotic nature, fungal cells have only a restricted set of unique targets. This makes it difficult to selectively target fungal cells. Two major families have been used for more than two decades to combat fungi; these include azoles that inhibit sterol formation and polyenes that bind to mature membrane sterols. However, the development of fluconazole resistance among different pathogenic strains and the high toxicity of amphotericin B (7-9) have prompted the studies of new antifungal agents with new modes of actions.

The investigation of antimicrobial peptides from a wide range of biological sources, and their synthetic derivatives, is a novel inroad to new antifungal agents. Antimicrobial peptides are gene-encoded and are part of the innate immunity to the microbial invasion of microorganisms of all types. The most studied group are short (<40 a.a.), positively charged, linear peptides whose possible mechanisms have been reviewed in detail (10-16). It is believed that most of these peptides are targeted to biological membranes, increase their permeability, and kill the cells. However, other additional mechanisms were proposed for some of them (17). Studies that investigated the mode of action of native antimicrobial peptides that are also endowed with antifungal activity are limited and included, for example, LL-37 and dermaseptines (18-20). These studies showed that they self-associate in solution and reach the membrane as oligomers (18-20).

Previously, we reported on a new approach for increasing the hydrophobicity and self-assembly in a solution of antibacterial peptides that are not active on fungi (21). We showed that peptide oligomerization in solution is crucial for the peptide to endow antifungal activity. In another study, we examined the effect of multiple substitutions of different amino acids regarding the structure, binding, and biological function of positively charged diastereomers (include both D- and L-amino acids) of linear lytic peptides (22). For this purpose, a group of diastereomeric peptides with the sequence K4X7W were synthesized, where X designates one of
the following aliphatic amino acids: Gly, Ala, Val, or Leu. The results revealed that the peptides containing Ala and Gly are not active against microorganisms concomitant with their inability to bind membranes. This is in agreement with the notion that a threshold of hydrophobicity along with a defined structure are required for antimicrobial activity. Here, we conjugated palmitic acid to this series of diastereomers and investigated their biological function and mode of action. Very interestingly, we found that the most potent antibacterial and antifungal lipopeptides are those derived from the peptides containing Ala and Gly. Studies on the function and structure of the peptides together with their interaction with bacteria, fungi, and model membranes, shed light on their mode of action. Furthermore, these studies demonstrated that a lipophilic tail can compensate for the hydrophobicity and amphipathic structure of the peptidic chain previously shown to be a prerequisite for antimicrobial activity.

MATERIALS AND METHODS

Materials. Rink amide MBHA resin and 4-Methyl benzhydrylamine resin (BHA) and 9-Fluorenylemethoxycarbonyl (F-moc) amino acids were obtained from Calbiochem-Novabiochem AG (Switzerland). Other reagents used for peptide synthesis include trifluoroacetic acid (TFA, Sigma), piperidine (Merck), N,N-diisopropylethylamine (DIEA, Sigma), N-hydroxybenzotriazole hydrate (HOBT, Aldrich), 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethylyuronium hexafluorophosphate (HBTU) and dimethylformamide (DMF, peptide synthesis grade, Biolab). Egg phosphatidylcholine (PC), phosphatidylinositol (PI) and phosphatidylethanolamine (PE), ergosterol were purchased from Lipid Products (South Nutfield, U.K.). 3-3′-dipropylthiadicarbocyanine-iodide (DiSC3-(5)), and Calcein were purchased from Molecular Probes (Junction City, OR). All other reagents were of analytical grade. Buffers were prepared in double distilled water. Amphotericin B and Trypsin (bovine pancreas) were purchased from Sigma Chemical Company (Israel). RPMI 1640 was purchased from Biological Industries (Beit Haemek, Israel). Proteinase K (fungal) was purchased from Beckman (Germany).
Peptide Synthesis, Acylation, and Purification. Peptides were synthesized by a F-moc solid phase method on Rink amide MBHA resin, using an ABI 433A automatic peptide synthesizer. The lipophilic acid was attached to the N-terminus of a resin-bound peptide using standard F-moc chemistry. Briefly, after removal of the F-moc from the N-terminus of the peptide with a solution of 20% piperidine in DMF, the fatty acid (seven equivalents (1M 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 by 95% trifluoroacetic acid (TFA) and were purified by RP-HPLC (reverse phase high-performance liquid chromatography) on a C18 (for non-lipidic peptides) or C4 (for the lipopeptides) Bio-Rad semi-preparative column (250x10 mm, 300Å pore-size, 5-µm particle size). The purified peptides were shown to be homogeneous (>99%) by analytical RP-HPLC. The elution time of the lipopeptides increased by ~20 min, indicating an increase in hydrophobicity owing 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 lipophilic acid conjugated analogs was performed according to the conditions of NCCLS 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 \(2 \times 10^3\) Colony-Forming Units (CFU)/ml in culture medium (RPMI 1640, 0.165 M MOPS, pH 7.0, with L-Glutamine, without NaHCO₃ medium) was added to 100 µl of water containing the peptide in serial 2-fold dilutions. The fungi were incubated for 24 h for opportunistic fungi or 48-72 h for \(C. albicans\) and \(C. neoformans\) at 35°C 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. The fungi used were \(Aspergillus fumigatus\) ATCC 26430, \(Aspergillus flavus\) ATCC 9643, \(Aspergillus niger\) ATCC 9642, \(Candida albicans\) ATCC 10231, and \(Cryptococcus neoformans\) ATCC MYA-422.
**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 10⁶ Colony-Forming Units (CFU)/ml in culture medium (LB medium) were added to 50 µl of water containing the peptide in serial 2-fold dilutions in water (prepared from a stock solution of 1 mg/ml peptide in water). 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 minimal inhibitory concentration (MIC), the concentration at which no growth was observed after 18-20 h of incubation. The bacteria used were *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Acinetobacter baumannii* ATCC 19606, *Bacillus subtilis* ATCC 6051, and *Staphylococcus aureus* ATCC 6538P.

**Hemolysis of Human Red Blood Cells (hRBC).** Fresh hRBC with EDTA were rinsed 3 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 hRBC 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 zero hemolysis (blank) and 100% hemolysis consisted of hRBC suspended in PBS and Triton 1%, respectively.

**Preparation of Liposomes.** Small unilamellar vesicles (SUV) were prepared by sonication as described earlier (23). Briefly, dry lipids were dissolved in CHCl₃: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 (7 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., 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 a mixture of zwitterionic phospholipids and
ergosterol (PC/PE/PI/ergosterol, 5:2.5:2.5:1 w/w), a lipid composition used to mimic the major components of the outer leaflet of Candida albicans (24).

Circular Dichroism (CD) Spectroscopy. The CD spectra of the lipopeptides were measured with a Aviv 202 spectropolarimeter. The spectra were scanned with a thermostatic quartz optical cell with a path length of 1 mm. Each spectrum was recorded in an average time of 5 sec, at steps of 0.2 nm at a wavelength range of 260 to 190 nm. The lipopeptides were scanned at a concentration of 10–100 µM in PBS (35 mM phosphate buffer, 0.15 M NaCl, pH 7.3) and 100 µM in the presence of 1% lysophosphatidylcholine (LPC) micelles. Fractional helicities (25,26) were calculated as follows:

$$\frac{[\theta]_{222} - [\theta]^0_{222}}{[\theta]^{100}_{222} - [\theta]^0_{222}}$$

where $[\theta]_{222}$ is the experimentally observed mean residue ellipticity at 222 nm, and values for $[\theta]^0_{222}$ and $[\theta]^{100}_{222}$, corresponding to 0% and 100% helix content at 222 nm, are estimated to be –2,000 and –32,000 deg.cm$^2$/dmol, respectively (25).

In Vivo Transmembrane Potential Depolarization Assay in Bacteria and Yeast. Membrane destabilization, which results in the collapse of transmembrane potential, was detected fluorimetrically by using a fluorescence dye (27) (see details below for bacteria and yeasts). The dye binds the plasma membrane owing to the cell transmembrane potential, resulting in a quenching of the dye's fluorescence. Peptide-induced membrane permeation caused a dissipation of the transmembrane potential that was monitored by an increase in fluorescence due to release of the dye. Experiments were performed in sterile 96-well plates (Nunc F96 microtiter plates) in a final volume of 100 µl, as follows: 50 µl of the cell suspensions and the dye were added to 50 µl of water containing the peptide in serial 2-fold dilutions. Membrane depolarization was monitored by an increase in the fluorescence of diS-C3-5 (excitation wavelength $\lambda_{ex} = 622$ nm, emission wavelength $\lambda_{em} = 670$ nm).
Transmembrane Potential Depolarization on the yeast C. neoformans. The assay was performed with intact yeast. More specifically, cells were centrifuged at 4000 g for 5 min at 4°C in a SS-34 rotor after being incubated at 35°C with agitation for 24 h in RPMI buffer (RPMI 1640, 0.165 M MOPS, pH 7.0, with L-Glutamine and without NaHCO₃). The cells were resuspended in PBS (without Ca²⁺ and Mg²⁺) to an inoculum of 4x10³ Colony-Forming Units (CFU)/ml. Cells were incubated with 1 µM diS-C₃-5 followed by fluorescence dequenching until a stable baseline was achieved (~ 50 min), indicating the incorporation of the dye onto the yeast membrane.

Transmembrane Potential Depolarization in E. coli. Spheroplasts of the Gram-negative bacteria E. coli D21 were prepared by the osmotic shock procedure (28). Bacteria were grown at 37°C with agitation to the mid log phase. Cells from cultures grown (O.D₆₀₀ = 0.8) were harvested by centrifugation and washed twice with 10 mM Tris/H₂SO₄, 25% sucrose, pH 7.5. Cells were resuspended in the washing buffer containing 1 mM EDTA. After 10 min of incubation at 20 °C with rotary mixing, the cells were collected by centrifugation and resuspended immediately in cold (0°C) water. After 10 min of incubation at 4 °C with rotary mixing, the spheroplasts were collected by centrifugation. The spheroplasts were then resuspended to O.D₆₀₀ of 0.05 in a buffer containing 20 mM glucose, 5 mM HEPES, 1 M KCl, pH 7.3. The cells were incubated with 1 µM diS-C₃-5, followed by fluorescence dequenching until a stable baseline was achieved (~ 2 h), indicating the incorporation of the dye into the bacteria membrane.

ATR-FTIR Spectroscopy. Spectra were obtained with a Bruker equinox 55 FTIR spectrometer equipped with a deuterated triglyceride sulfate (DTGS) detector and coupled with an ATR device, after removing the trifluoroacetate (CF₃COO⁻) counterions, as has been described in detail in other studies (21). The sample was hydrated by exposure of the samples to an excess of deuterium oxide (D₂O). The H/D exchange was considered complete due to the complete shift of the amide II band. Any contribution of D₂O vapor to the absorbance spectra near the amide I peak region was eliminated by subtraction of the spectra of pure lipids equilibrated with D₂O under the same conditions.
**ATR-FTIR Data Analysis.** To resolve overlapping bands, we processed the spectra using PEAKFIT™ (Jandel Scientific, San Rafael, CA) software. Second-derivative spectra were calculated to identify the positions of the component bands in the spectra. These wavenumbers were used as initial parameters for curve fitting with gaussian component peaks. Positions, bandwidths, and amplitudes of the peaks were varied until a good agreement between the calculated sum of all components and the experimental spectra was achieved ($r^2 > 0.999$). The relative amounts of different secondary structure elements were estimated by dividing the areas of individual peaks assigned to a particular secondary structure by the whole area of the resulting amide I band.

**Analysis of the Polarized ATR-FTIR Spectra.** The ATR electric fields of incident light were calculated as follows (29,30).

\[
E_x = \frac{2 \cos \theta \sqrt{\sin^2 \theta - n_{21}^2}}{\sqrt{(1-n_{21}^2)(1+n_{21}^2)\sin^2 \theta - n_{21}^2}}
\]

\[
E_y = \frac{2 \cos \theta}{\sqrt{1-n_{21}^2}}
\]

\[
E_z = \frac{2 \sin \theta \cos \theta}{\sqrt{(1-n_{21}^2)(1+n_{21}^2)\sin^2 \theta - n_{21}^2}}
\]

where $\theta$ is the angle of a light beam to the prism normal at the point of reflection (45°), and $n_{21} = n_2/n_1$ ($n_1$ and $n_2$ are the refractive indices of ZnSe, taken as 2.4, and the membrane sample, taken as 1.5, respectively). Under these conditions, $E_x$, $E_y$, and $E_z$ are 1.09, 1.81, and 2.32, respectively. The electric field components, together with the dichroic ratio (defined as the ratio between absorption of parallel (to a membrane plane), $A_{\parallel}$, and perpendicularly polarized incident light, $A_{\perp}$) are used to calculate the orientation order parameter, $f$, by the following formula:
where \( \alpha \) is the angle between the transition moment of the amide I vibration of an \( \alpha \)-helix and the helix axis. Several values ranging from 27°-40° were reported in the literature for \( \alpha \) (31). We used the values of 27° (29,32) and 39° (33) for \( \alpha \). Lipid order parameters were obtained from the symmetric (~2853 cm\(^{-1}\)) and antisymmetric (~2922 cm\(^{-1}\)) lipid stretching modes using the same equations, setting \( \alpha = 90° \) (29).

**Tryptophan Fluorescence Measurements.** To determine the environment of the peptides, we measured changes in the intrinsic tryptophan fluorescence in PBS and upon binding to vesicles. A peptide (1 \( \mu \)M) was added to PBS, or PBS containing 1 mM PC/PE/PI/ergosterol (5:2.5:2.5:1) SUVs. Emission spectra were measured on a SLM-Aminco, Series 2 Spectrofluorimeter, with the excitation set at 280 nm, using a 4-nm slit, recorded in the range of 300–400 nm (4-nm slit). In these studies, SUVs were used to minimize differential light-scattering effects, (34) and the lipid/peptide molar ratio was kept high (1000:1), so spectral contributions of free peptide would be negligible.

**RESULTS**

**Peptide's Design and Sequences.** Four diastereomeric peptides were synthesized corresponding to the sequence KX3KWX2KX2K (X=Gly, Ala, Val, or Leu). They were designed to create a perfect amphipathic \( \alpha \)-helical structure in their L-form, as revealed by the Schiffer & Edmundson wheel projection (35). Each diastereomer contained four D-amino acids at positions 3, 4, 8, 10, when X=Ala, Val, or Leu, or alternatively at positions 1, 5, 9, 12, when X=Gly. A Trp was introduced in each peptide to serve as an intrinsic fluorescent probe. All four peptides were amidated at their C-terminus and conjugated at their N-terminus to palmitic acid (PA), a linear saturated chain of 16 carbons (CH\(_3\)-(CH\(_2\))\(_{14}\)-COOH). Table 1 shows the sequence of the peptides, their designations and molecular weights.
**Antibacterial and Antifungal Activity.** Antibacterial activity was assayed against three strains of Gram negative (P. aeruginosa, A. baumannii, and E. coli) and two strains of Gram positive (S. aureus, B. subtilis) bacteria. Table 2 presents the MIC of the peptides against the tested bacteria. The data revealed that both D-V and its palmitoylated analog were devoid of antibacterial activity. However, in contrast to D-V, D-L was highly active toward all bacteria tested, but its palmitoylated analog was also devoid of antibacterial activity. Most interestingly, whereas both D-G and D-A were devoid of antibacterial activity, their palmitoylated analogs PA-D-G and PA-D-A were highly active toward all the bacteria tested.

We further tested the antifungal activity of the peptides against representative pathogenic yeasts and fungi. The list includes Candida albicans, Cryptococcus neoformans, Aspergillus fumigatus, Aspergillus flavus, and Aspergillus niger. The results are summarized in Table 3. The data revealed that in all cases palmitoylation significantly increased the antifungal activity. Most surprisingly, similar to the antibacterial activity of PA-D-G and PA-D-A are the most active lipopeptides toward all the tested fungi and yeasts, whereas the more hydrophobic lipopeptides, PA-D-V and PA-D-L, are only partially active.

**Hemolytic activity of the lipopeptides.** The hemolytic activity of the lipopeptides against a highly diluted solution of human erythrocytes (4%) is shown in Figure 1. The data revealed that PA-D-G and PA-D-A, which were highly active against bacteria, yeasts, and fungi showed low hemolytic activity. In contrast, PA-D-V and PA-D-L, which were practically devoid of antibacterial activity and only partially killed yeasts and fungi, were highly hemolytic.

**Oligomerization of the Lipopeptides in Solution Detected by Tryptophane Fluorescence and CD Spectroscopy.** We monitored the fluorescence emission spectrum of the intrinsic tryptophan in aqueous solution. We found that the signal of the Trp emission in PA-D-V was fully quenched (Table 4) possibly because of close proximity between the tryptophans which causes self-quenching. Since tryptophan is located along the peptide chain, we can assume that the peptidic moiety exists as an oligomer in solution, as suggested previously for the non-lipidic form (22).
The data also revealed a significant blue shift in the case of PA-D-L, which suggests that the peptide is located in a more hydrophobic environment. This could possibly happen if the peptide interacts with the lipid moiety, either by bending on itself, as suggested previously with the all L-amino acid magainin derivative (21), or alternatively, because of the formation of micelles in which the peptide is bound to the lipidic moiety. In contrast, no blue shift was observed for PA-D-G and PA-D-A, which suggests that these lipopeptides are either monomeric or oligomerize through the lipidic moiety, whereas the Trp environment is exposed to the solution.

To further support our findings, we measured the effect of the lipophilic moiety on the structure of the peptides in PBS as measured by CD spectroscopy (Figure 2). Dose-dependent (from 10 µM to 100 µM) CD spectral profiles of the lipopeptides showed that only PA-D-L adopts a well-defined \( \alpha \)-helical structure, which is concentration-independent. A peptide can form a distinct secondary structure in solution either if it oligomerizes such that the hydrophobic surfaces are packed one against another and the hydrophilic surfaces face the solution, or alternatively, if it is inserted into a hydrophobic environment that could be produced by micellization of the palmitoyl groups. K,L containing diastereomeric peptides were shown previously to be monomeric in solution (22). Furthermore, the blue shift in Trp fluorescence (see above) revealed that the peptide is located in a hydrophobic environment. These data support the second possibility, suggesting the formation of mixed micelles.

**Secondary Structure of the Lipopeptides in the Membrane.** The secondary structure of the lipopeptides was measured in PC/PE/PI/ergosterol multibilayers by using ATR-FTIR spectroscopy. We exchanged hydrogen with deuterium (see Materials and Methods) to make it possible to differentiate \( \alpha \)-helical components from a random structure, since the absorption of the random structure shifts to a greater extent than the \( \alpha \)-helical component after deuteration (36). The amide I region spectra of the bound lipopeptides are shown in Figure 3. Second derivatives accompanied with Savitsky-Golay smoothing were calculated to identify the positions of the component bands in the spectra and are given in the corresponding panels in Figure 3. We used these wavenumbers as initial parameters for curve fitting with gaussian component peaks. Table 5 summarizes the assignments, wavenumbers (\( \nu \)), and relative areas of
the component peaks. The assignment of the different secondary structures to the various amide I regions was calculated according to the values taken from Jackson and Mantsch (37). The data reveal that the major amide I band of PA-D-V is located at ~1627 cm\(^{-1}\) (78±1%), corresponding to the aggregated β-sheet, whereas that of PA-D-L has two peaks at 1635 cm\(^{-1}\) (44%) and 1652 cm\(^{-1}\) (32%), suggesting the existence of both β-sheet and α-helical structures. In contrast, both PA-D-G and PA-D-A are predominantly random coils.

**Orientation of the Phospholipid Membrane and the Effect of the Lipopeptides on the Phospholipid Acyl-Chain Order.** Polarized ATR-FTIR spectroscopy was used to determine the orientation of the lipid membrane. The symmetric \([\nu_{\text{sym}}(\text{CH}_2) \approx 2853 \text{ cm}^{-1}]\) and the antisymmetric \([\nu_{\text{asym}}(\text{CH}_2) \approx 2922 \text{ cm}^{-1}]\) vibrations of lipid methylene C-H bonds are perpendicular to the molecular axis of a fully extended hydrocarbon chain. Thus, measurements of the dichroism of infrared light absorbance can reveal the order and orientation of the membrane sample relative to the prism surface. However, since the intensity of the antisymmetric CH\(_2\) vibration was higher than one, our calculations were based only on R values taken from the symmetric vibration. The data revealed that the membranes are predominantly in a liquid-crystalline phase (R=1.33), indicating that the lipid multibilayers used were well-oriented (29,38). The effect of the lipopeptides on the multibilayer acyl-chain order can be estimated by comparing the CH\(_2\)-stretching dichroic ratio of pure phospholipid multibilayers and membrane-bound lipopeptides. The data indicate that the highest effect on the lipid order was obtained with PA-D-V (R=1.55), whereas the incorporation of all the other lipopeptides into the membrane changed the lipid order to a much lesser extent (R=1.44), suggesting they are surface-localized.

**In Vivo Transmembrane Potential Depolarization**

(i) *Transmembrane Potential Depolarization on the Yeast C. neoformans.* In order to test the ability of the lipopeptides to permeate the microorganism cell wall, we monitored the collapse of the transmembrane potential in living yeast in the presence of the lipopeptides by using a potential sensitive dye. All the lipopeptides were able to dissipate the membrane potential of *C.*
C. neoformans to a high extent (Figure 5, panel A). Interestingly, these results correlate well with the antifungal activity of the different peptides against C. neoformans. However, there is a slight difference between the MIC and the concentration of peptides required to dissipate 100% of the yeast transmembrane potential. This might be due to differences in the conditions used for the antifungal assay and the potential depolarization assay. These results suggest that the target of the lipopeptides is the plasma membrane of the cells.

(ii) Transmembrane Potential Depolarization of E. coli Spheroplasts. Since the cytoplasmic membrane of bacteria is negatively charged compared with the zwitterionic membrane of fungi, we performed potential depolarization assays using spheroplasts of the Gram-negative bacteria E. coli D21. A dose-response curve of the lipopeptide-mediated potential depolarization activity is shown in Figure 5, panel B. All the lipopeptides are highly active in disrupting the membrane potential of the E. coli D21 strain. However, a good correlation between the antibacterial activity and the potential to permeate the cytoplasmic membrane was found only with PA-D-G and PA-D-A. The findings that both PA-D-V and PA-D-L are highly active on spheroplasts but not on intact bacteria and that both form large oligomers may indicate that they are not active on E. coli because they are not able to penetrate through the cell wall into the cytoplasmic target membrane.

DISCUSSION

The most interesting result in this study is the finding that the attachment of palmitic acid to the N-terminus of positively charged short peptides, which are inert toward microorganisms (D-G, D-A peptides), endowed them with a broad spectrum of potent antibacterial and antifungal activity, and with a low hemolytic activity against the highly diluted solution of hRBCs. In contrast, the same modification to a potent antimicrobial peptide (D-L peptide), abolished its antibacterial activity, but increased both its antifungal and hemolytic activities. Furthermore, the parental D-V peptide was inactive toward bacteria, fungi and yeasts, whereas its palmitoylated form was active only toward the two strains of yeasts tested (Tables 2,3).
Many studies with membrane-active peptides have demonstrated the important role of hydrophobicity and structure for their biological function (39,40). Here, all four parental peptides were derived from a similar peptidic backbone and contain the same number of positive charges located at the same positions. However, they differ markedly in their hydrophobicities. We show that palmitic acid, attached to the N-terminus of the inactive peptides, can compensate for the hydrophobicity of the peptidic chain. Note that shorter acyl analogues could enhance the antibacterial activity of a peptide fragment of human lactoferrin, which originally had weak antibacterial activity (41), or endowed antibacterial peptides with antifungal activity (21).

The peptidic moieties have different structures and organizations within the lipopeptide’s oligomers

In order to shed light on the mode of action of these new lipopeptides, we examined their oligomeric state, their ability to increase the membrane permeability, as well as their structure and location when bound to membranes. Previous studies showed that the parental non-palmitoylated peptides differ markedly (i) in their ability to bind and permeate phospholipid membranes, (ii) in their structure in the membrane, and (iii) in the oligomeric state in solution and membranes (22). These studies have shown that D-G and D-A peptides are inert toward both zwitterionic and negatively charged phospholipid membranes, and are monomeric in solution. In contrast, both D-V and D-L peptides bound strongly and permeated both types of phospholipid membranes. However, whereas D-V forms oligomers in solutions and membranes, D-L is monomeric. In addition, all the parental peptides were also found to be unstructured in solution.

Here, all the palmitoylated peptides are expected to form micelles. Indeed, previous studies showed that the micellar concentration of palmitic acid conjugated to an amino acid is in the µM range and should significantly decrease by increasing the peptidic chain (42). However, the structure and the organization of the peptidic chains in solution are different for the lipopeptides. CD spectroscopy revealed a defined α-helical structure in solution only for PA-D-L (Figure 2). Furthermore, the Trp environment of this peptide is more hydrophobic than that expected in solution (Table 4). This may happen if the peptidic chain interacts with the micelles formed by the palmitic moiety. Regarding PA-D-V, the finding of strong quenching of the Trp
supports the notion, that in addition to the micelles formed by the palmitic group, the peptidic chain of PA-D-V is also aggregated. In contrast, the relatively hydrophilic environment of the Trp in PA-D-G and PA-D-A suggests that their peptidic moiety is not aggregated and not intercalated within the palmitic micelles. This in agreement with the findings that these two parental peptides bind only weekly to lipids (22).

The role of lipopeptide’s oligomerization on target-cell specificity

Previous studies have shown that the peptide's oligomerization is a characteristic of antimicrobial peptides that act on zwitterionic phospholipids, which are abundant in the plasma membrane of variable eukaryotic organisms from fungi to humans (18,19,43). Because of this, such peptides have both antifungal and hemolytic activities. However, whereas reversible oligomerization has been shown to be important for antibacterial and antifungal activity, it was not crucial for hemolytic activity, because covalently linked pentamers of antimicrobial peptides became highly hemolytic (44). Here all four lipopeptides form oligomers in solution. However, in two of them, the Val and Leu lipopeptides, the peptidic chains stabilize the oligomers; in PA-D-V there is peptide-peptide interaction, and in PA-D-L, peptide-palmitoyl interaction. Therefore, the dissociation of these two oligomers is probably more difficult than the dissociation of the lipopeptides containing Gly and Ala, once they bound to the target cell.

Oligomer dissociation seems to be an important requirement for antimicrobial activity, because many pathogens including bacteria, yeasts, and fungi are surrounded, in addition to the plasma membrane, by an external barrier, which contains mainly polysaccharide compounds. Therefore, to reach the cytoplasmic phospholipid membranes (the possible target of the lipopeptides, as will be discussed in the following section), they need to traverse the microorganism's cell wall. As soon as the peptides cross the cell wall of the pathogen, the only barrier left is the plasma membrane. Thus, the smaller the size of the molecule, the higher is its ability to penetrate through the cell wall to reach the plasma membrane. This should result in improved biological activity. Hancock and coworkers termed this process for Gram-negative bacteria, a self-promoted uptake (45). Indeed, the most active and those with the broadest spectrum of activity are PA-D-G and PA-D-A. However, both are also the less hemolytic
peptides. This can be explained if we take into account the negatively charged glycocalix layer surrounding the cytoplasmic membrane of erythrocytes. The positively charged peptides are probably attached first to the glycocalix layer via electrostatic interaction. However, because the peptidic moiety has low affinity to zwitterionic membranes, it makes it more difficult for these two peptides to partition into the cell phospholipid membrane, compared with the more hydrophobic ones.

**The plasma membrane is the main target of the lipopeptides**

Similar to other antimicrobial peptides, the main target of the lipopeptides is the plasma membrane. This is supported by the finding of a correlation between the antifungal activity of the lipopeptides against *C. neoformans* and their ability to disrupt the membrane potential of the intact yeast (Figure 5, Panel A). In addition, when we removed the cell wall of *E. coli* (by preparing spheroplasts), all the lipopeptides possessed high cell-permeating activity (Figure 5, Panel B) which correlates with the hydrophobicities of the peptides. We have also performed studies by using model phospholipid membranes. These studies included: (i) measuring the environment of the Trp residue when bound to vesicles; (ii) determining the effect of the four lipopeptides on the acyl chain order; and (iii) determining the secondary structure of the lipopeptides within the phospholipid membranes. The data revealed that three lipopeptides PA-D-G, PA-D-A and PA-D-L, had a strong and similar effect on the lipid order, as well as the extent of blue shift of the Trp. This is true despite differences in their secondary structures. However, PA-D-V was an exception, because it disrupted the lipid order to a greater extent and no fluorescence signal could be detected from its Trp. This is probably because the peptide is aggregated and the Trp fluorescence is fully quenched. This was confirmed by ATR-FTIR spectroscopy that showed that PA-D-V forms mostly β-aggregates in the membrane, whereas PA-D-L adopted a predominantly helix/dynamic helix, and PA-D-G and PA-D-A adopted random coils.

Note that native lipopeptides already exist and they possess a broad spectrum of activities, including antibacterial, antifungal, antiviral, and cytolytic activities (46-50) (reviewed in (51)). However, compared with the lipopeptides presented here, most of them have a unique
alkyl chain bound to a short peptide chain (6-7 amino acids), and are cyclic (52), and composed mainly of hydrophobic and acidic L- and D-amino acids. They have been shown to act either via non-specific membrane lysis, e.g., iturin (53) and surfactin (54), or via inhibition of the synthesis of cell wall components, e.g., the echinocandins family, which inhibits the synthesis of 1,3-β-D-glucan (55) (reviewed in (9)). However, although the echinocandins, especially caspofungin, were approved by the FDA for therapeutic usage (56,57) they are limited mostly to Candida and Aspergillus species whereas the Cryptococcal disease remains uncovered by these drugs. Furthermore, their ability to act on a specific target makes them more amendable to develop resistance. The biophysical studies presented here together with the findings of a direct correlation between the MIC, and the in vitro and in vivo transmembrane potential depolarization assays, suggest that our lipopeptides act directly on the cell plasma membrane and not on a specific target. Furthermore, previous studies revealed that the position of the D-amino acids of a diastereomeric peptide can be changed to produce a repertoire of new active compounds that differ from each other by their structure in solution (and hence should have different antigenicities), and by their susceptibility to enzymatic degradation (58). Controlled enzymatic degradation is an advantage because lytic peptides that are fully degraded or fully protected from enzymatic degradation are less suitable for therapy. Further studies on this new family of lipopeptides, which are highly active toward both bacteria and C. neoformans, should assist in the development of broad-spectrum antifungal agents.
REFERENCES


Table 1: Peptides’ sequences and designations

<table>
<thead>
<tr>
<th>Peptide Designation</th>
<th>Peptide Formula</th>
<th>Sequence</th>
<th>Molecular weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA-D-G</td>
<td>PA-D-K(^{1,5,9,12})-K(_4)G(_7)W</td>
<td>(CH(<em>3</em>)-(CH(<em>2</em>))(_{14})-CO)-\textbf{KGGGKWGGKGGK}-NH(_2)</td>
<td>1353</td>
</tr>
<tr>
<td>PA-D-A</td>
<td>PA-D-A(^{3,4,8,10})-K(_4)A(_7)W</td>
<td>CH(<em>3</em>)-(CH(<em>2</em>))(_{14})-CO)-\textbf{KA\textsubscript{AA}KWA\textsubscript{AA}KA}-NH(_2)</td>
<td>1451</td>
</tr>
<tr>
<td>PA-D-V</td>
<td>PA-D-V(^{3,4,8,10})-K(_4)V(_7)W</td>
<td>(CH(<em>3</em>)-(CH(<em>2</em>))(_{11})-CO)-\textbf{KV\textsubscript{YY}KWVVKYVK}-NH(_2)</td>
<td>1647</td>
</tr>
<tr>
<td>PA-D-L</td>
<td>PA-D-L(^{3,4,8,10})-K(_4)L(_7)W</td>
<td>(CH(<em>3</em>)-(CH(<em>2</em>))(_{14})-CO)-\textbf{KL\textsubscript{LL}KWLLKL}-NH(_2)</td>
<td>1746</td>
</tr>
</tbody>
</table>

Bold and underlined letters are D-amino acids. The C-terminus of all the peptides is amidated. PA denotes palmitic acid.
Table 2: Minimal Inhibitory Concentration (MIC) of the peptides against bacteria

<table>
<thead>
<tr>
<th>Peptide designation</th>
<th>Minimal Inhibitory Concentration (µM)</th>
<th>Gram-positive bacteria</th>
<th>Gram-negative bacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><em>S. aureus</em> (ATCC 6538P)</td>
<td><em>B. subtilis</em> (ATCC 6051)</td>
</tr>
<tr>
<td>D-G</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>PA-D-G</td>
<td>12.5</td>
<td>3.12</td>
<td>6.25</td>
</tr>
<tr>
<td>D-A</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>PA-D-A</td>
<td>12.5</td>
<td>3.12</td>
<td>6.25</td>
</tr>
<tr>
<td>D-V</td>
<td>&gt;100</td>
<td>60</td>
<td>&gt;100</td>
</tr>
<tr>
<td>PA-D-V</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>D-L</td>
<td>30</td>
<td>1.25</td>
<td>7.5</td>
</tr>
<tr>
<td>PA-D-L</td>
<td>&gt;100</td>
<td>n.d.</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

For peptides’ sequences see Table 1.
n.d. – not determined
Table 3: Minimal Inhibitory Concentration (MIC) of the peptides against yeasts and fungi

<table>
<thead>
<tr>
<th>Peptide Designation</th>
<th>Yeast</th>
<th>Fungi</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>Candida albicans</em> (ATCC 10231)</td>
<td><em>Cryptococcus neoformans</em> (ATCC MYA-422)</td>
</tr>
<tr>
<td>D-G</td>
<td>$&gt;100$</td>
<td>$&gt;100$</td>
</tr>
<tr>
<td>PA-D-G</td>
<td>3.125</td>
<td>6.25</td>
</tr>
<tr>
<td>D-A</td>
<td>$&gt;100$</td>
<td>$&gt;100$</td>
</tr>
<tr>
<td>PA-D-A</td>
<td>12.5</td>
<td>1.56</td>
</tr>
<tr>
<td>D-V</td>
<td>$&gt;100$</td>
<td>50</td>
</tr>
<tr>
<td>PA-D-V</td>
<td>3.125</td>
<td>3.125</td>
</tr>
<tr>
<td>D-L</td>
<td>25</td>
<td>3.125</td>
</tr>
<tr>
<td>PA-D-L</td>
<td>$&gt;100$</td>
<td>3.125</td>
</tr>
</tbody>
</table>

For peptides' sequences see Table 1.
Table 4: Tryptophan emission maxima (in nm) of the lipopeptides in aqueous solution (PBS) or in the presence of PC/PE/PI/ergosterol (5:2.5:2.5:1 w/w) vesicles

<table>
<thead>
<tr>
<th>Peptide designation</th>
<th>PBS</th>
<th>Vesicles&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA-D-G</td>
<td>351±1</td>
<td>336±1</td>
</tr>
<tr>
<td>PA-D-A</td>
<td>349±1</td>
<td>335±1</td>
</tr>
<tr>
<td>PA-D-V</td>
<td>n.d.&lt;sup&gt;b&lt;/sup&gt;</td>
<td>n.d.&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>PA-D-L</td>
<td>340±1</td>
<td>334±1</td>
</tr>
</tbody>
</table>

n.d. – not detected.

<sup>a</sup> A lipid-to-peptide molar ratio of 1000:1 was used in all cases.

<sup>b</sup> no signal was detected.
Table 5: Secondary structure of the lipopeptides in PC\PE\PI\ergosterol (5:2.5:2.5:1 w/w) multibilayer

<table>
<thead>
<tr>
<th>Peptide designation</th>
<th>Peptide assignment</th>
<th>Aggregated β-sheet (1620-1628 cm⁻¹)</th>
<th>β-sheet (1628-1640 cm⁻¹)</th>
<th>Random coil (1640-1645 cm⁻¹)</th>
<th>Random coil/α-helix (1645-1650 cm⁻¹)</th>
<th>α-helix (1650-1654 cm⁻¹)</th>
<th>Dynamic/310-helix (1655-1670 cm⁻¹)</th>
<th>β-sheet/Turn (1670-1680 cm⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA-D-G</td>
<td></td>
<td>1644±1 65%±1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1662±1 22%±1</td>
<td>1677±1 13%±1</td>
</tr>
<tr>
<td>PA-D-A</td>
<td></td>
<td>1644±1 91%±1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1664±1 4%±2</td>
<td>1677±1 5%±2</td>
</tr>
<tr>
<td>PA-D-V</td>
<td></td>
<td>1627±1 78%±1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1649±1 12%±1</td>
<td></td>
</tr>
<tr>
<td>PA-D-L</td>
<td></td>
<td>1635± 44%±1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1652± 32%±1</td>
<td>1666± 16%±1</td>
</tr>
</tbody>
</table>

The results are the average of four independent experiments.

\(^a\) A 1:60 peptide:lipid molar ratio was used.
FIGURE LEGENDS

Figure 1: Dose-response of the hemolytic activity of the lipopeptides toward hRBC. The assay was performed as described in the Materials and Methods section. The designations are as follows: PA-D-G (△); PA-D-A (□); PA-D-V (▲); PA-D-L (■). The results are the mean of three separate experiments.

Figure 2: CD spectra of the lipopeptides in PBS. Spectra were taken at a lipopeptide concentration of 100 µM. The assay was performed as described in the Materials and Methods section. The designations are as follows: PA-D-G (△); PA-D-A (□); PA-D-V (▲); PA-D-L (■).

Figure 3: FTIR spectra of the fully deuterated amide I band (1600 to 1700 cm⁻¹) and the corresponding second derivative of the lipopeptides in PC/PE/PI/ergosterol (5:2.5:2.5:1 w/w) multibilayers. The sum of the fitted components (thick line) and the fitted components (thin line) of the lipopeptides are presented in the upper panel of each scheme. Second derivatives were calculated to identify the positions of the component bands in the spectra and are presented in the lower panel of each scheme. The designations are as follows: PA-D-G (panel A), PA-D-A (panel B), PA-D-V (panel C), and PA-D-L (panel D). All curves represent the experimental FTIR spectra after Savitzky-Golay smoothing. A 60:1 lipid/diastereomer molar ratio was used.

Figure 4: CD spectra of the lipopeptides in LPC micelles. Spectra were taken at a lipopeptide concentration of 100 µM in ddw, containing 1% of LPC. The assay was performed as described in the Materials and Methods section. The designations are as follows: PA-D-G (△); PA-D-A (□); PA-D-V (▲); PA-D-L (■).

Figure 5: Maximal dissipation of the diffusion potential induced by the lipopeptides by using intact yeast and E. coli D21 spheroplasts. The lipopeptides were added to C. neoformans conidia (Panel A) or E. coli D21 spheroplasts (Panel B) that were pre-incubated with the fluorescent dye diS-C₃-5 for 5 min. Membrane depolarization was monitored by an increase in the fluorescence of diS-C₃-5 (excitation wavelength λₑₓ = 622 nm, emission
wavelength $\lambda_{em} = 670$ nm) after the addition of peptides at different concentrations. Fluorescence recovery was measured as a function of time with 5-10 min of intervals and its maxima was reported. The designations are as follows: PA-D-G (△); PA-D-A (□); PA-D-V (▲); PA-D-L (■).
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
A new group of antifungal and antibacterial lipopeptides derived from non-membrane active peptides conjugated to palmitic acid
Dorit Avrahami and Yechiel Shai

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