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Supramolecular Complexes of Ultrashort Cationic Lipopeptides with Cyclodextrins: Improved Selectivity and Therapeutic Potential

Chiara Bellini^{1,2}  | Unai Atxabal³  | Szilvia Bősze^{4,5} | Orsolya Dobay⁶ | Andrea Horváth⁶ | Imola Cs. Szigártó⁷ | Tamás Beke-Somfai⁷ | Jesús Jiménez-Barbero³ | István Puskás⁸ | Kata Horváti¹ 

¹MTA–HUN–REN TTK Lendület “Momentum” Peptide-Based Vaccines Research Group, Institute of Materials and Environmental Chemistry, HUN–REN Research Centre for Natural Sciences, Budapest, Hungary | ²Hevesy György PhD School of Chemistry, Eötvös Loránd University, Budapest, Hungary | ³Center for Cooperative Research in Biosciences (CIC bioGUNE), Basque Research and Technology Alliance (BRTA) Derio, Bizkaia, Spain | ⁴HUN–REN–ELTE Research Group of Peptide Chemistry, Hungarian Research Network, Budapest, Hungary | ⁵Department of Genetics, Cell- and Immunobiology, Faculty of Medicine, Semmelweis University, Budapest, Hungary | ⁶Institute of Medical Microbiology, Semmelweis University, Budapest, Hungary | ⁷Biomolecular Self-assembly Research Group, Institute of Materials and Environmental Chemistry, HUN–REN Research Centre for Natural Sciences, Budapest, Hungary | ⁸CycloLab Cyclodextrin Research and Development Lab. Ltd., Budapest, Hungary

Correspondence: Kata Horváti (horvati.kata@ttk.hu)

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ABSTRACT

In the last decade, the rise of antibiotic resistance has heightened interest in antimicrobial peptides and lipopeptides as promising alternatives to conventional antibiotics because of their lower propensity to develop resistance. However, lipopeptides often show undesired cytotoxicity due to their non-selective membrane disruptive effect, and their limited aqueous solubility represents a matter of concern from a pharmaceutical point of view. This study demonstrates a panel of ultrashort cationic lipopeptides (USCLs) consisting of a tetrapeptide (L1), originated from buforin II, coupled with saturated fatty acids of different lengths. Our results highlight that the 16-carbon fatty acid lipopeptide (Pal-L1) exhibits relevant antibacterial activity against multiresistant *Staphylococcus aureus* strain. However, the formation of heterogenic aggregates in cell culture medium and toxic effects on human cells were also observed. Pal-L1 formulation with the randomly methylated α -cyclodextrin (RAMEA) and the sulfobutylether- β -cyclodextrin (SBECD) has resulted in a production of ultralow-sized molecular dispersion systems and reduced lipopeptide toxicity without compromising its antimicrobial activity. With titration ¹H-NMR, 2D NMR experiments, together with molecular dynamics simulations, we described the size, structure, stoichiometry, and dissociation constant of the supramolecular complexes. Interactions of neutral and negatively charged model liposomes with Pal-L1 lipopeptide in the presence or absence of cyclodextrins serve an explanation for the membrane selectivity, and based on the results, we proposed a potential mechanism of action for the Pal-L1+cyclodextrin complexes on different biological membranes. Overall, our model characterization points out that cyclodextrin formulation improves the therapeutical applicability of lipopeptides.

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1 | Introduction

The World Health Organization (WHO) declared antimicrobial resistance (AMR) one of the top 10 global public health threats, leading to 10 million annual deaths by 2050 [1]. Although the development of resistance is an evolutionary response by microorganisms, key determinants have expedited this process over time, like the overuse and misuse of antibiotics in humans and animals, as well as the presence of suboptimal preventive measures, such as the absence of effective vaccines [2, 3]. Designing better therapeutics is especially urgent when vaccines are unavailable, as in the cases of *Pseudomonas aeruginosa*, *Burkholderia*, and *Staphylococcus aureus*, or when the existing vaccine does not provide appropriate protection, like in the case of *Mycobacterium tuberculosis*. In recent years, there has been a reduction in the release of new antibiotics on the market, and the current clinical pipeline primarily focuses on improving existing molecules [4, 5]. While newly developed derivatives have frequently demonstrated increased effectiveness, they typically share the same target as their precursors, leading to the quick emergence of cross-resistance [6]. The preclinical pipeline looks more diverse and innovative. While the focus on direct-acting small molecules is still predominant, it also includes non-traditional approaches such as antimicrobial peptides (AMPs) [7].

Currently, the Antimicrobial Peptide Database (APD3) contains almost 4000 AMPs, including ones that have received approval for human use, like LL-37, polymyxin B, and daptomycin [8]. Despite their diversity in sequence and structure, most AMPs share some properties; they are usually short in length (<100 amino acids), cationic, and amphipathic molecules [9]. They can exhibit different mechanisms of action, although their activity is directed primarily toward the bacterial membrane and mainly controlled by charge and hydrophobicity [10, 11]. While the positive charge is crucial for the initial interaction with the negatively charged microbial membranes, the hydrophobic domain promotes the peptide permeation and the perturbation of the membrane integrity [12].

Natural AMPs can serve as templates for the rationale design of synthetic analogs or derivatives [13, 14]. In the design, the hydrophobic-to-charge ratio and amphiphilicity must be carefully addressed, as they are the major factors determining the selectivity for bacterial cells [15]. Moreover, the original scaffolds can be optimized by different chemical modifications, such as incorporating non-natural amino acids or D-amino acids, peptide cyclization, glycosylation, and lipidation [16–19]. Lipidation can enhance the effectiveness of natural AMPs or confer antibacterial properties onto biologically inactive peptides [19, 20]. For instance, fatty acid conjugation to a peptidyl scaffold is a promising approach to develop potent antimicrobial agents [21–25]. On the other hand, lipopeptides can have limited solubility in aqueous solutions and exhibit toxicity in human cells, including hemolytic activity.

Cyclodextrins (CDs) are a family of α -1,4-linked cyclic oligosaccharides consisting of either six (α -), seven (β -), or eight (γ) glucose units. They have a toroidal-like shape characterized by a hydrophilic outer surface and a more hydrophobic central cavity. This peculiar arrangement enables them to form inclusion

complexes with molecules of appropriate size and geometry. Hence, they are frequently used as excipients in pharmaceutical formulations to increase the aqueous solubility of poorly soluble active substances, enhance the stability and bioavailability of drugs, and reduce potential undesired side effects, such as toxicity [26–28].

In this study, we designed a panel of synthetic ultrashort cationic lipopeptides (USCLs) by coupling saturated fatty acids of different lengths to a cationic peptide (L1). This 4-mer peptide scaffold was derived from a classical AMP, namely, buforin II that was isolated from the stomach tissue of the Asian toad *Bufo bufo gargarizans* [29]. A structure–activity relationship study revealed that the C-terminal Leu-Leu-Arg-Lys tetrapeptide has an important role in pore-forming activity, and the deletion of this sequence resulted in a complete loss of antimicrobial effect [30]. However, the Leu-Leu-Arg-Lys (L1) tetrapeptide itself is inactive against bacteria and fungi.

Our primary aim was to identify the optimal length of the alkyl chain to observe an antibacterial effect. The 16-carbon fatty acid derivative, Pal-L1, proved to be a promising candidate to target *S. aureus*. However, it exhibited significant cytotoxicity in human cells and poor aqueous solubility. Therefore, we investigated the use of different CDs to target Pal-L1 drawbacks. Our results proved that peptide formulation with randomly methylated α -cyclodextrin (RAMEA) and sulfobutylether- β -cyclodextrin (SBECD) decreased the undesired hemolytic side effects of the lipopeptide toward bacterial cells, and increased its solubility in physiological solutions.

2 | Results and Discussion

2.1 | USCLs Panel Design, Synthesis, and Analytical Characterization

Due to its simple structure and cationic nature, the tetrapeptide L1 (Leu-Leu-Arg-Lys) was a suitable scaffold for developing short antimicrobial lipopeptides. Indeed, arginine and lysine can establish electrostatic interactions with negatively charged bacterial membranes. In addition, the guanidinium group of arginine can form extensive hydrogen bonds with the phospholipid head groups in the membranes [31].

Achieving the optimal degree of hydrophobicity is crucial for maximizing the activity of AMPs. Previous studies on lipidated AMPs identified the optimal chain lengths ranging from 8 to 12 carbons across different bacteria, such as *Escherichia coli*, *P. aeruginosa*, and *S. aureus* [19, 32]. In the specific context of antimicrobial USCLs, longer chain fatty acids, such as 14- and 16-carbon fatty acids, often resulted as the most active compounds [24, 33–35]. However, it is noteworthy that there is a threshold in the relationship between hydrophobicity and antibacterial activity, as longer fatty acid derivatives can demonstrate toxicity toward mammalian cells and an increased tendency for aggregation, ultimately leading to a reduction in antibacterial effectiveness [19].

In this study, L1 was synthesized on solid phase using Fmoc/^tBu strategy and a panel of USCLs was obtained by coupling saturated

TABLE 1 | Analytical characterization of the **L1** and the panel of USCLs.

Peptide	Sequence	M _{mo} Meas. ^a	RT (min) ^b	Log P ^c	Log D ^d at pH 7.4	Calc. solubility at pH 7.4 (mg/mL) ^e
L1	LLRK	527.3879	7.0	−1.83	−7.35	>200
Val-L1	CH ₃ (CH ₂) ₃ CO - LLRK	611.4463	11.3	−0.47	−5.14	>200
Dec-L1	CH ₃ (CH ₂) ₈ CO - LLRK	681.5213	15.1	1.75	−2.91	137
Myr-L1	CH ₃ (CH ₂) ₁₂ CO - LLRK	737.5873	14.1*	3.53	−1.13	3.40
Pal-L1	CH ₃ (CH ₂) ₁₄ CO - LLRK	765.6170	16.3*	4.42	−0.25	0.56
Ste-L1	CH ₃ (CH ₂) ₁₆ CO - LLRK	793.6485	19.0*	5.31	0.64	0.09

^aMonoisotopic molecular mass measured on a Thermo Scientific Q Exactive Focus Hybrid Quadrupole-Orbitrap Mass Spectrometer.

^bRetention time on Phenomenex Jupiter C12, gradient: 5%–100% B, 20 min; *40%–100% B, 20 min. According to the RP-HPLC analysis, the purity of the peptides was always above 95%. The C-terminus of the peptides was amidated.

^cLog P is the 10-base logarithmic measure of the partition coefficient, which is a ratio of concentrations of an un-ionized compound in the two phases of immiscible solvents (water and *n*-octanol) at equilibrium.

^dOctanol-water distribution coefficient at neutral pH, calculated by the Chemicalize program (<https://chemicalize.com>).

^eDetermined by Chemaxon's Solubility Predictor (<https://chemaxon.com>).

fatty acids of varying length (from C5 to C18) at the *N*-terminus of the peptide. The peptides were characterized by analytical RP-HPLC and mass spectrometry (Table 1 and Supporting Information S1 and Figures S1–S6). All the lipopeptides were amidated at their C-terminus. The calculated isoelectric point of the USCL panel is 11.9 and at neutral pH and the lipopeptides bear net two positive charges. The retention time was used as an index of the peptide hydrophobicity, and a linear increase of the measured parameter was observed as a function of the fatty acid length. Calculated parameters such as Log P (partition coefficient) and Log D (distribution coefficient at pH 7.4 in octanol-water) also showed good correlation with the fatty acid length, as shown in Table 1. The solubility was calculated at pH 7.4. Note that for Pal-L1, the calculated solubility was 0.56 mg/mL at pH 7.4, but 35.2 mg/mL at pH 6.5.

2.2 | Antibacterial Activity of USCLs

The antibacterial activity of L1 and its fatty acid derivatives was investigated by determining their minimum inhibitory concentration (MIC) against reference strains of methicillin-susceptible (MSSA) and methicillin-resistant (MRSA) *S. aureus*, and Gram-negative bacteria *E. coli* and *P. aeruginosa* (Table 2). The 14-carbon fatty acid was the critical threshold required to observe potent antimicrobial activity, near 10 μM. Shorter fatty acid derivatives affected neither Gram-positive nor Gram-negative bacteria. Myr-L1 demonstrated the broadest spectrum of activity, effectively targeting both *S. aureus* strains as well as Gram-negative bacteria. Longer fatty acid derivatives maintained the activity against *S. aureus* with Pal-L1 showing comparable efficacy to Myr-L1, while Ste-L1 was somewhat less active. Our results align with previous studies proving that 14- and 16-carbon fatty acid derivatives of short cationic peptides were the most suitable lengths to have activity against *S. aureus* [24, 33, 35, 36].

The higher sensitivity of Gram-positive bacteria to antibacterial lipopeptides has been previously documented, and it probably arises from the different cell wall composition compared to Gram-negative bacteria [22, 37, 38]. Antibacterial lipopeptides

typically target the cytoplasmic membrane of Gram-positive bacteria, resulting in membrane disruption, depolarization, and subsequent loss of intracellular components. The initial step of the antibacterial action is proposed to be the interaction of the positively charged peptide part with the negatively charged phospholipids within the cytoplasmic membrane. Consequently, the lower abundance of negatively charged phospholipids in Gram-negative bacteria appears to play a role in the bacterial selectivity of cationic lipopeptides [37]. Furthermore, the additional outer membrane of Gram-negative bacteria, which is the hallmark of these species, serves as a permeability barrier for hydrophobic compounds and at the same time allow the entry of small hydrophilic molecules such as nutrients. The molecular basis for this lies in the composition of the outer membrane as a unique asymmetrical bilayer, with phospholipids in the inner leaflet and lipopolysaccharide (LPS) facing outward. The strong lateral interactions between the LPS molecules stabilize the outer membrane of Gram-negative bacteria and prevent the passage of hydrophobic molecules. This may explain our observation that the more hydrophobic Pal-L1 and Ste-L1 do not act on Gram-negative species, while the less hydrophobic Myr-L1 does. Thus, the lack of activity of Pal-L1 and Ste-L1 could be associated with their significant hydrophobicity, which may prevent them from penetration through the cell wall of the Gram-negative bacteria [39].

2.3 | USCLs Toxicity on Human Cells

The lack of selectivity and the resulting toxicity in human cells are among the main concerns when using receptor-independent antimicrobial agents, such as USCLs. Thus, the potential toxicity of the synthesized lipopeptides was tested in human MonoMac6 (MM6) monocytic cell line, a widely accepted model system for investigating the immunological and genetic functions of human monocytes/macrophages and for studying compounds' toxicity to mature blood monocytes [40]. The results highlighted that the treatment with L1 and the short fatty acid derivatives did not affect the cell viability up to 100 μM, while Myr-L1, Pal-L1, and Ste-L1 showed relevant toxicity (Table 2). In the case of

TABLE 2 | The minimum inhibitory concentration and IC₅₀ of the USCL panel.

	<i>Staphylococcus aureus</i> ATCC 29213 (MSSA)	<i>S. aureus</i> ATCC 33591 (MRSA)	<i>Escherichia coli</i> ATCC 25922	<i>Pseudomonas aeruginosa</i> ATCC 27853	Human MM6 cells	Human red blood cells
	MIC (μ M) ^a			IC ₅₀ (μ M) ^b		HC ₅₀ (μ M) ^c
L1	>100	>100	>100	>100	>100	>100
Val-L1	>100	>100	>100	>100	>100	>100
Dec-L1	100	100	>100	>100	>100	>100
Myr-L1	12.5	12.5	25	50	22.8	21.4
Pal-L1	12.5	12.5	>100	>100	10.6	12.6
Ste-L1	25	25	>100	>100	6.1	10.8

Note: The antibacterial activity of L1 and the fatty acid derivatives was determined after 24-h treatment using the serial microdilution method.

^aMinimal inhibitory concentration (MIC) value was defined as the lowest concentration of the compound that inhibits the growth of the bacteria.

^bHalf maximal inhibitory concentration (IC₅₀) value was defined as the peptide concentration that caused the 50% reduction in viability of MonoMac-6 human monocytes after 24-h treatment.

^cHC₅₀ values were defined as the peptide concentration that caused 50% reduction in human red blood cell viability after 2-h treatment. Data are reported as mean values of 4 parallels.

these compounds, a correlation between fatty acid length and cytotoxicity was observed (Figure S7A). Indeed, a higher Log *p*-value was associated with a lower IC₅₀ value (Tables 1 and 2).

The USCL panel showed varying degrees of hemolytic activity and follows the order of the previously presented cytotoxicity results, namely, the L1, Val-L1 and Dec-L1 peptides were harmless to the human red blood cells up to 100 μ M concentration, but in the case of longer lipid tails, serious hemolysis was observed (Figure S7A). The HC₅₀ values of Myr-L1, Pal-L1, and Ste-L1 were found to be very close to the IC₅₀ values (Table 2 and Table S1), suggesting that the membrane rupture effect of the lipopeptides occurs by a similar mechanism in monocytes and in red blood cells.

For further studies, Pal-L1 was selected because of its lack of selectivity and the limited aqueous solubility, which makes it a reliable and exciting model to tackle typical challenges within this group of molecules.

2.4 | Pal-L1 Solubility in Aqueous Media

Hydrophobicity not only impacts the antimicrobial effectiveness and cytotoxicity of USCLs, but also their aqueous solubility. Indeed, it is well-established that long fatty acids, such as palmitate, are poorly soluble in water and physiological buffers [41]. Consequently, the tendency of lipopeptides to aggregate could limit their applicability as pharmaceutical agents. For example, the reduced physical stability influences the selection of the administration route, and can also give rise to other significant issues, such as toxicity, reactogenicity, and loss of activity [42].

Sterilization by filtration is a suitable method for biopharmaceuticals that cannot undergo traditional heat or chemical sterilization methods, such as protein and peptide-based therapeutics. It is

also frequently used to decrease bioburden levels in process streams, preventing the potential formation of biofilms. However, when peptide or protein solutions are exposed to liquid–solid interfaces, as in the case of membrane filtration, physical loss of the compound can be observed. The loss can occur through processes like membrane adsorption or due to alterations in the conformational structure of the protein or peptide, such as aggregation [43]. Looking at the more specific context of this study, peptide aggregation during sterile filtration before proceeding with in vitro and in vivo studies represents a significant concern, as it can affect the quality and reliability of the experimental results. Hence, we investigated the potential Pal-L1 aggregation in aqueous media and the associated risk of peptide loss during membrane filtration.

After method validation (Table S2 and Figure S8), the peptide solubility in water was evaluated by RP-HPLC after membrane filtration (cutoff of 200 nm). The area under the curve (AUC) obtained from the filtrate was then compared to the AUC of Pal-L1 dissolved in the internal control (0.1% TFA in AcN:water 80:20 [v/v]), which perfectly solubilizes the lipopeptide. Our results proved that approximately 40% of the peptide was contained in the filtrate, when water was used for the solubilization. At the same time, the concentration dramatically decreased when Pal-L1 was dissolved in physiological solvents, such as PBS and RPMI cell culture media (Figure 1A). This result is in line with the calculated pH-dependent solubility values (Table 1) that were 35.2 mg/mL at pH 6.5 but only 0.56 mg/mL at pH 7.4.

The unfiltered Pal-L1/PBS sample contains particles that are clearly visible to the eye (Figure 1B), and when examined under a microscope, aggregated fibrils of different sizes and shapes were observed (Figure 1C). Indeed, the larger size of the aggregates, compared to the monomeric peptide molecules, can result in a more robust and extensive interaction with the cell membranes, potentially leading to a higher disruptive effect on the membrane bilayer [44].

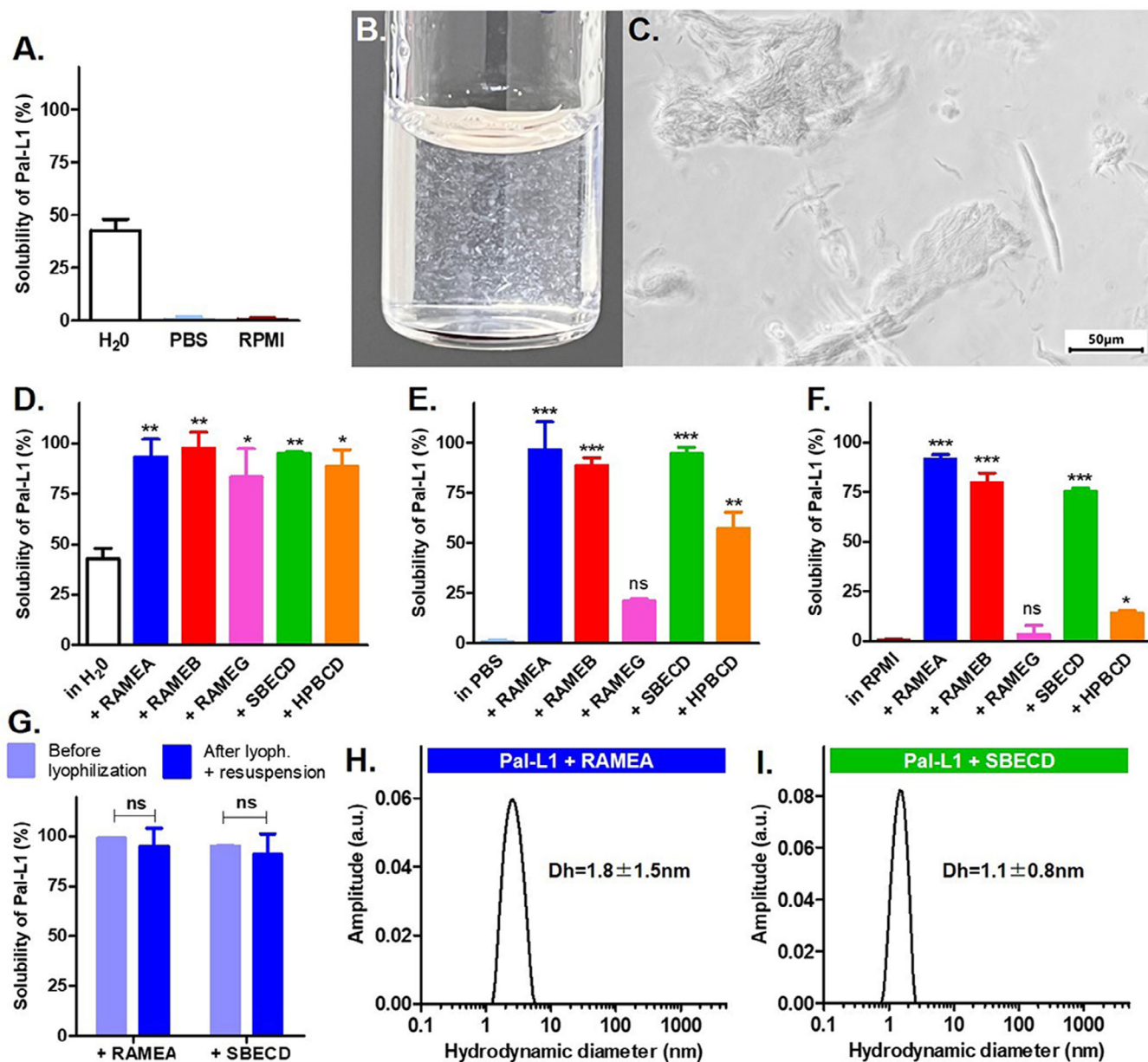


FIGURE 1 | Solubility of Pal-L1 and CD-formulated Pal-L1 in water, PBS, and cell culture media. The solubility of Pal-L1 was studied using RP-HPLC after filtration (A). The soluble peptide concentration (%) was expressed comparing the area under the curve (AUC) of Pal-L1 obtained after filtration to the AUC of the peptide dissolved in 0.1% TFA AcN:water (80:20 v/v). Representative photograph (B) and microscopic image (VWR IT415PH, 40×) (C) are captured of the visible aggregates of Pal-L1 in PBS. Pal-L1 (100 μM) was formulated with 5 mM CDs in aqueous solution, and then further diluted in water (D), PBS (E), and RPMI (F) to investigate the complex solubility with RP-HPLC. Chemical stability of the complex was studied before and after freeze-drying. For the reconstitution of the lyophilizates, d.i. H₂O was used (G). Each bar is representative of two parallel measurements ± SD. The AUC of formulated Pal-L1 was compared to the corresponding internal control, and statistical analysis was performed using one-way ANOVA, followed by Tukey's post hoc test; statistical significance: ****p* < 0.001; ***p* < 0.01; **p* < 0.1; ns: not significant. Size and size distribution of the Pal-L1-cyclodextrin complex was evaluated by dynamic light scattering (DLS) and the mean hydrodynamic diameter (Dh) ± SD (*N* = 10) was determined (H and I).

2.5 | Cyclodextrin Formulation of Pal-L1 and its Effect on the Peptide Solubility

Different alpha-, beta-, and gamma-cyclodextrin derivatives were probed for the formulation of Pal-L1 lipopeptide (Table 3). The applied cyclodextrins, modified with substituents such as methyl-, sulfolbutylether and hydroxypropyl groups, are different in terms of cavity size, charge, and hydrophobic character. These

substituents can promote or inhibit the complexation based on their characteristics and modify the behavior of the host-guest inclusion complexes.

The preliminary screening pointed out that all applied CDs at a concentration of 50 mM significantly improved the aqueous solubility of Pal-L1, except the HPGCD (Figure S9). The solubilization efficacy of CDs was maintained using a 10-fold lower

TABLE 3 | List of the used cyclodextrins

Abbreviation	Name	Average Mw
RAMEA	Randomly methylated α -cyclodextrin	1140.9
RAMEB	Randomly methylated β -cyclodextrin	1303.1
RAMEG	Randomly methylated γ -cyclodextrin	1465.6
SBECD	Sulfobutylether- β -cyclodextrin	2163.3
HPBCD	2-hydroxypropyl- β -cyclodextrin	1396.5
HPGCD	2-hydroxypropyl- γ -cyclodextrin	1396.5

cyclodextrin concentration, and the peptide recovery rate was above 80% in all cases (Figure 1D). The results were in agreement with previous outcomes suggesting the higher complexation efficacy of fatty acids by α - and β -CDs over γ -CDs [45]. Based on the cavity size, α -CD and its derivatives, especially RAMEA, were previously advised to be the most appropriate complexing partners for saturated fatty acids with a length between 10 and 18 carbons [46]. Moreover, our findings aligned with supporting evidence demonstrating the effective complexation of fatty acids with RAMEB, SBECD, and HPBCD in water [45].

Our previous observations indicated a higher propensity for the lipopeptide to aggregate in a media, better modeling physiological environment compared to a water solution. As shown in Figure 1E, this tendency was substantially reduced in PBS by the complexation of Pal-L1 with RAMEG and HPBCD, while the inclusion complex formation was almost similar in the case of RAMEA, RAMEB, and SBECD. The reduced peptide recovery with HPBCD and RAMEG in PBS might be due to a lower affinity for Pal-L1 compared to the other CDs. Despite the host-guest interaction strength being adequate for observing complexation in water, it proved insufficient to counteract the higher tendency of the peptide to aggregate in PBS.

The CD complexation of Pal-L1 was also investigated in RPMI, mimicking the experimental conditions used in the in vitro cell viability studies. In this case, only RAMEA, RAMEB, and SBECD proved to solubilize the peptide efficiently (Figure 1F). Conversely, the efficacy of RAMEG and HPBCD drastically decreased, with less than 15% of Pal-L1 recovered after the membrane filtration.

Next, we evaluated the peptide-recovery both before and after freeze-drying process to assess the stability of Pal-L1 complex with RAMEA and SBECD. As shown in Figure 1G, no significant difference of this parameter was observed after the complex lyophilization and resuspension in water.

The host-guest inclusion complexes were also examined by dynamic light scattering (DLS) and the results showed small particles of about 1–2 nm in diameter (Figure 1H,I). In addition, no particles of the size typical of Pal-L1 lipopeptide were seen and the solution was clear. A diameter of around 1 nm is a typical size for cyclodextrin monomers, measured for them in the concentration range below the critical aggregation concentration (cac) [47]. The cac for the cyclodextrins used in this study is in between 1.7% and 2.5% (m/v) [47], which is in all cases higher than the concentration range we used for the lipopeptides.

2.6 | Cyclodextrin Formulation of Pal-L1 and its Effects on Cytotoxicity, Hemolytic, and Antimicrobial Activities

First, we did a screen for the cyclodextrin using 100 μ M Pal-L1 and 50 *equiv.* of CD (5 mM). As shown in Figure 2A,B, the cytotoxicity and hemolysis results were overall consistent with the evidence from the solubilization study. A significant improvement in the cell viability was observed when Pal-L1 was formulated with RAMEA and SBECD, which previously demonstrated enhanced peptide solubility and complexation. Conversely, HPBCD and RAMEG failed to solubilize Pal-L1 effectively and did not reduce its cytotoxic and hemolytic activity.

This result proved so promising that we tested this effect on other human model cells, and found that both HT29 human colorectal and HepG2 human liver cells gave the same response, that is, the RAMEA and SBECD cyclodextrins abolished the membrane disrupting effect of Pal-L1 in all cell types (Figure 2C–F). For these experiments, only 20 *equiv.* of cyclodextrins was applied for the solubilization of Pal-L1. Interestingly, the IC₅₀ values were very close for all cell types, suggesting that the Pal-L1 lipopeptide may act in the same way on all mammalian cells, regardless of their tissue origin.

As control, we also tested the cytotoxic effect of the cyclodextrins alone, as shown in Figure S10, and found that the cyclodextrins were not cytotoxic and hemolytic up to the highest tested concentration (5 mM), except RAMEB.

It is widely known that RAMEB has high affinity for membrane cholesterol, leading to increased membrane binding and, consequently, cytotoxicity at higher concentrations [48]. Also, the extraction of cholesterol by RAMEB inhibits the clathrin-dependent endocytic pathway, which is crucial for a rapid internalization of essential molecules, such as hormones and growth factors [49].

In summary, RAMEA and SBECD proved to be the most promising cyclodextrins to target Pal-L1 toxicity, and this cytoprotective effect was independent of the cell type.

We also investigated whether the formulation with RAMEA and SBECD could affect the antibacterial activity of Pal-L1. While SBECD did not affect the activity of Pal-L1 against Gram-positive or Gram-negative strains, RAMEA formulation has resulted in a somewhat improved antibacterial activity of the peptide against *S. aureus* strains (Figure 3A,B). Such as, the MIC value decreased

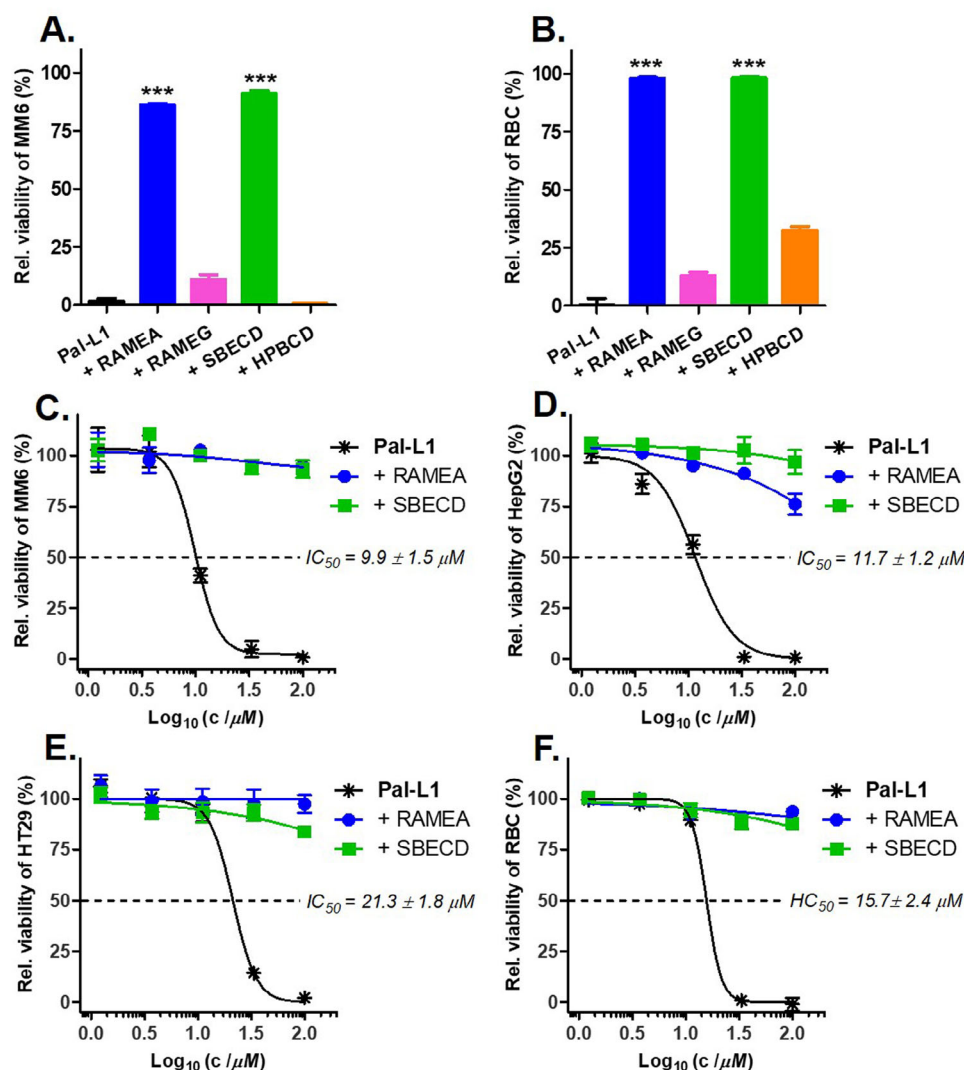


FIGURE 2 | In vitro cytotoxic effect of Pal-L1 and Pal-L1 cyclodextrin complexes. Cyclodextrins were screened by adding 5 mM CD and 100 μM Pal-L1 lipopeptides to the cells, and then viability of MonoMac-6 human monocytes (A) and human red blood cells (B), relative to the medium-treated control cells, was determined. For the more detailed in vitro characterization (C–F), Pal-L1 was measured alone, or in the presence of 20 equiv. cyclodextrins on MonoMac-6 human monocytes (C), HepG2 human hepatocytes (D), HT29 human colorectal cells (E), and on human red blood cells (F). Data are presented as the mean of four parallels ± SD. Statistical analysis was performed using one-way ANOVA, followed by Tukey's post hoc test; statistical significance: *** $p < 0.001$.

from 12.5 to 6.25 μM both against the methicillin-susceptible and methicillin-resistant bacteria. As control experiment, we also tested the bacterial growth in the presence of cyclodextrins, and not significant but a slight decrease was measured for the RAMEA but just in the case of the MSSA strain (Figure S11). On the Gram-negative strains (*E. coli* and *P. aeruginosa*), none of the compounds showed antibacterial effect up to 100 μM, with or without CDs.

It is worth noting that while we observed significant differences in cytotoxicity and hemolytic activity between CD-formulated and non-formulated lipopeptides on mammalian cells, we did not experience this difference in the antibacterial activity. We speculate that this may be due to differences in the structure and composition of the mammalian cell membrane and the cell wall of the bacteria. To gain deeper insight into the

cause of the observed peculiar behavior of the complexes, a titration experiment was performed to investigate how different CD/lipopeptide ratios affect the antibacterial activity and the cytotoxicity, respectively. We found that the antibacterial effect of the Pal-L1+cyclodextrin complex was very close to that of the unformulated lipopeptide's activity, irrespectively to the used CD concentration, while the undesired hemolytic activity followed a concentration-dependent trend having reduced toxicity with increasing CD/lipopeptide ratio (Table 4). Based on the experimentally determined correlation, we can define an optimal concentration range for the cyclodextrins that minimizes the cytotoxic side effects, but maintains the antibacterial activity. The selectivity of Pal-L1 was significantly higher when RAMEA was applied higher than 10 equiv. quantity, while in the case of SBECD 5 equiv. was sufficient to improve the selectivity index of the lipopeptide.

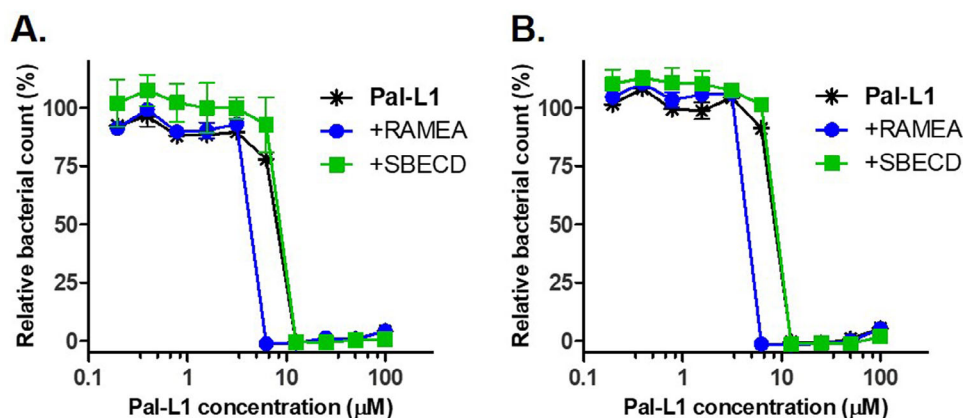


FIGURE 3 | Antibacterial effect of the Pal-L1 lipopeptide and its CDs complexes. In the highest tested concentration, 100 μM Pal-L1 peptide was formulated with 5 mM CDs, and then two-fold serial dilution was prepared in Mueller Hinton Broth. The relative bacterial count was measured on reference strains of methicillin-susceptible (MSSA) (A) and methicillin-resistant (MRSA) (B) *Staphylococcus aureus* using standard broth microdilution method. Data are mean $\pm$ SD ($N = 2$). Based on the results, the minimal inhibitory concentration (MIC) values can be calculated, which is 12.5 μM for the Pal-L1 peptide and for the Pal-L1+SBECD complex, while 6.25 μM for the Pal-L1+RAMEA complex.

TABLE 4 | Titration of the biological effect of Pal-L1 lipopeptide in the presence of cyclodextrins at different concentrations.

	CD/Pal-L1 ratio	MIC (μM) ^a	HC ₅₀ (μM) ^b	SI ^c
Pal-L1	—	12.5	13.7	1.1
+ RAMEA	1	12.5	18.7	1.5
	5	6.25	30.5	4.9
	10	6.25	61.2	9.8
	20	12.5	>100	>8.0
	10	25.0	>100	>8.0
+ SBECD	1	25.0	25.6	1.0
	2.5	12.5	37.8	3.0
	5	12.5	>100	>8.0
	10	25.0	>100	>4.0

Note: The antibacterial activity of L1 and the fatty acid derivatives was determined after 24-h treatment using the serial microdilution method.

^aMinimal inhibitory concentration (MIC) value was defined as the lowest concentration of the compound that inhibits the growth of the bacteria.

^bHalf maximal inhibitory concentration (IC₅₀) values were defined as the peptide concentration that caused 50% reduction in human red blood cell viability after 2-h treatment. Data are reported as mean values of 4 parallels.

^cThe selectivity index (SI) was calculated by the ratio of the hemolytic concentration (HC₅₀) to the minimal inhibitory concentration (MIC).

To study this phenomenon together with the observed difference in the effect against Gram-positive and Gram-negative bacteria, biophysical measurements were performed on model liposomes.

2.7 | Study of Pal-L1 and Pal-L1+CDs Complex on Liposomes

To monitor the effect of the Pal-L1 peptide and its cyclodextrin complexes (Pal-L1+RAMEA and Pal-L1+SBECD), DLS measurements were performed on model liposomes. Lipopeptide binding to the pure PC liposomes, which is neutral in charge, induced

no detectable changes in the correlation functions (Figure S12), while an increase in the average size of liposomes (from ~119 to 133 nm) was detected (Table S3). The changes may result from the incorporation of the peptide into the lipid bilayer and a weak interaction between the lipids and Pal-L1.

In contrast, for the two-component, negatively charged lipid system (PCPG), a shift in the autocorrelation function to higher decay times was observed when the liposomes were mixed with the Pal-L1 peptide (Figure S13), which refers to the formation of large-sized aggregates of the PCPG lipids (~0.5 to 1.5 μm) (Table S4). The effect was more pronounced after 24-h incubation time, where the estimated lipid aggregate size was in the micrometer range, accompanied with a high polydispersity (Table S4). Pal-L1 and Pal-L1+RAMEA bindings are probably more favored due to the electrostatic attraction between the peptide and PG head group, while the negatively charged SBECD buries the liposome destructive effect of Pal-L1.

Control measurements were also performed (Figures S14 and S15 and Tables S5 and S6) for the peptide-free systems, which confirmed the affinity of liposomes to interact with cyclodextrins (RAMEA and SBECD) mainly through the inclusion of the fatty acid chains in the cavity, without disruption of vesicles, which is also shown by the slight increase in the size of the liposomes.

The marked differences between the peptide interactions of the neutral PC and the negatively charged PCPG liposomes may account for the membrane selectivity of the Pal-L1 peptide. While only a weak interaction was observed for the neutral PC liposomes, significantly higher membrane affinity was measured for the negative PCPG liposomes, which is thought to contribute to Pal-L1's preference for binding to the cell wall of Gram-positive bacteria that is richer in anionic lipids [50].

Our observations are in line with the findings of Lin and Grossfield [51] who performed rigorous free energy calculations on palmitoylated KGGK tetrapeptide, bearing two cationic side chains, just as our Pal-L1 molecule. They aimed to uncover the

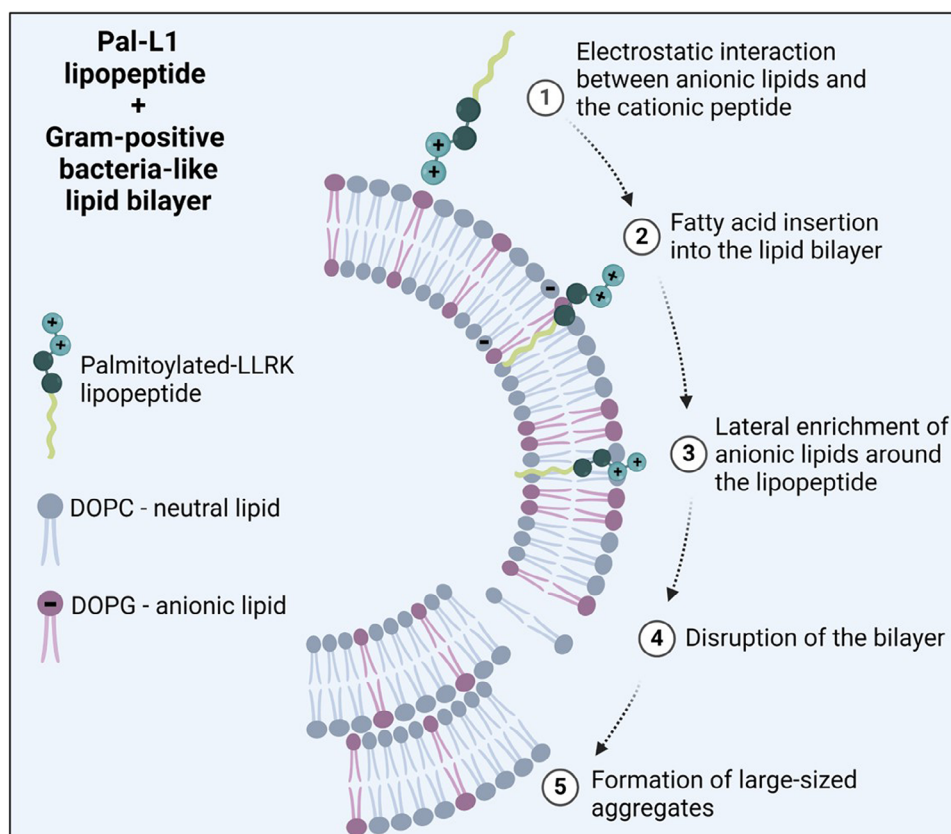


FIGURE 4 | Putative mechanism of action of Pal-L1 lipopeptide and negatively charged model PCPG liposomes, based on coarse-grained molecular dynamics simulations and free energy calculations of Lin and Grossfield [51] and our observations.

thermodynamic parameters influencing both membrane affinity and selectivity for bacterial versus mammalian membranes using coarse-grained molecular dynamics simulations and free energy methods. Data suggest that the C16 fatty acid chain contributes primarily to binding to the lipid membrane, while the peptide moiety is responsible for selectivity thanks to the electrostatic interaction between the cationic peptide and the anionic bacterial membrane, which contributes approximately 40% to the overall free energy (ΔG) of binding. The putative mechanism of action describes the first step as the peptide adsorption on the membrane surface via the lysin moieties interacting with the phosphate groups followed by an approximately 180° turn and the translocation of the fatty acid tail into the membrane. Then, as Lin and Grossfield show, membrane components in the vicinity of the lipopeptide become more organized and the lateral enrichment of the negatively charged lipids is suggested, which stabilizes its binding to the membrane [51]. This stabilization and energy gain can contribute to the thermodynamic origin of the membrane selectivity of cationic lipopeptides. Our experimental data together with the above-mentioned calculations inspired Figure 4 that represents the putative mechanism of membrane activity of Pal-L1 lipopeptide toward negatively charged lipid bilayers.

2.8 | Affinity Determination by ^1H -NMR

In the following, we were interested in the structure of the lipopeptide-CD supramolecular complexes. Titration ^1H -NMR

experiments were used, and the chemical shift perturbations of the CD NMR signals were monitored upon systematic additions of Pal-L1. In all cases, the CDs were maintained at 1 mM concentration (in 500 μL deuterated sodium phosphate buffer solution), while increasing concentrations of Pal-L1 were subsequently added until reaching 1:2 equivalents of CD/Pal-L1 ratio.

During the initial steps of the titration with RAMEA, no precipitation was observed, strongly suggesting that the lipopeptide was solubilized by the CD. However, when a 1:2 CD/Pal-L1 ratio was reached, precipitation was observed in the NMR tube, indicating the presence of free lipopeptide. For RAMEA, the addition of Pal-L1 originated selective shifts of H3 and H5, as well as 2-OMe and 3-OMe groups, while 6-OMe did not present any shift around them as a consequence of establishing non-covalent interactions (Figure 5A). The chemical shift perturbations in the ^1H -NMR of the most perturbed peaks (2- and 3-OMe) were plotted versus the peptide concentration, allowing to estimate a dissociation constant of 0.42 ± 0.12 mM as illustrated in Figure 5B. Additionally, a Job's plot of continuous variation [52] was represented (the chemical shift perturbations vs. the molar fraction $[X_{\text{RAMEA}}]$ upon addition of Pal-L1). The maximum point in the y axis was achieved in between the range X_{RAMEA} 0.5–0.6 (Figure 5C), strongly suggesting that the formed complex has a 1:1 stoichiometry ($A + B \rightarrow AB$).

Next, the ability of SBECD to form a complex with Pal-L1 was tested. Significant chemical shift perturbations (CSP) were also observed, especially for the NMR signals corresponding to

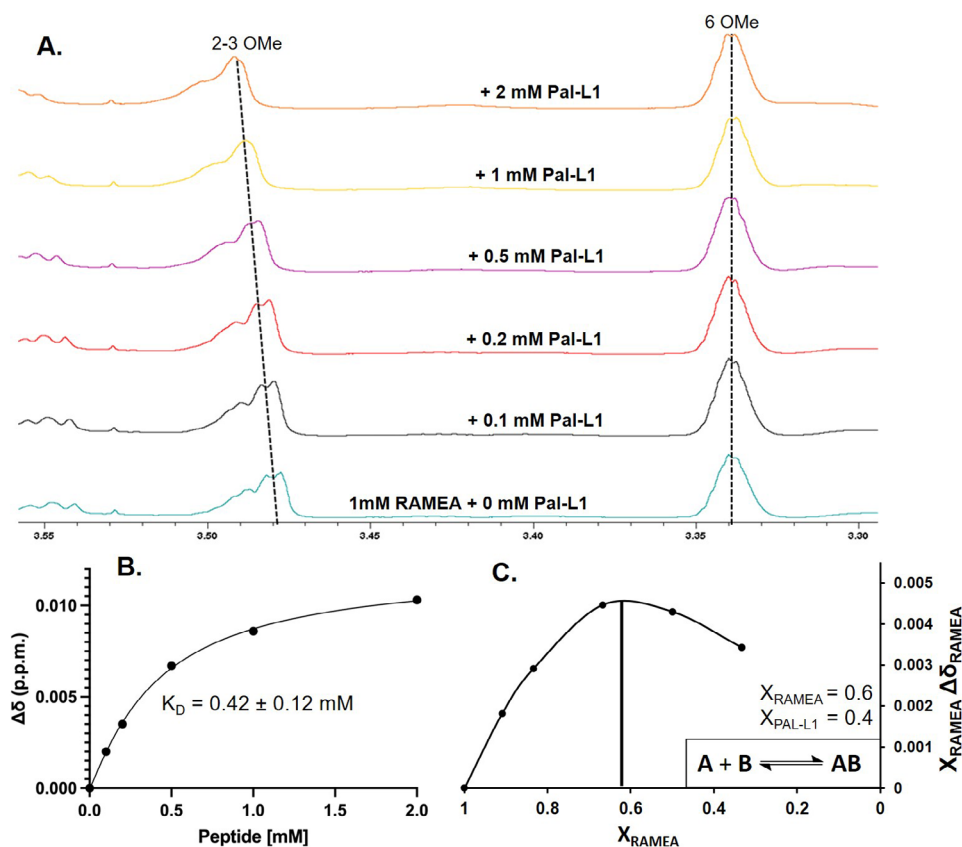


FIGURE 5 | ^1H -NMR titration between RAMEA (1 mM) and Pal-L1. The peaks of RAMEA are selectively shifted upon increasing concentrations of the lipopeptide. The peak shift of 2- and 3-OMe (ppm) versus peptide concentration (in mM) is plotted and fitted (A). The dissociation constant (K_D) obtained is illustrated on panel (B). Job's plot of the shifted ^1H -NMR resonances of RAMEA throughout complexation with Pal-L1 is presented on panel (C).

H5/H6 and the $\alpha\text{-CH}_2$ of the $n\text{-Bu-SO}_3$ scaffold. This evidence is indicative that intermolecular contacts are taking place between this region of the CD and the ligand (Figure 6A), different of those described above for RAMEA. In contrast, the NMR signals corresponding to $\delta\text{-CH}_2$ (next to SO_3), $\beta\text{-CH}_2$, and $\gamma\text{-CH}_2$ did not suffer any CSP. The estimated dissociation constant (K_D) was 0.15 ± 0.04 mM in this case (Figure 6B), while the Job's plot showed an X_{SBECD} of 0.67 as the highest intensity point, suggesting a SBECD/Pal-L1 complex of 1:2 stoichiometry (Figure 6C). Thus, two lipopeptide molecules fit within the cavity of SBECD ($A + 2B \rightarrow AB_2$). It should be noted that oligomerization and the observed high K_D values would prevent further quantification using this approach; however, the qualitative conclusions above still provide useful practical insights.

For the RAMEG, no CSPs were observed upon titration with Pal-L1 (Figure S16). Interestingly, for RAMEA and SBECD, NMR signals corresponding to the lipopeptide progressively appeared during the titration. In contrast, for RAMEG, those signals did not appear, while precipitates were observed in the NMR tube during the titration. This evidence suggests that no complex formation takes place in this case.

Regarding the HPBCD, CSPs were not observed during the titration. As in the previous case, no signals of the lipopeptide were observed, which indicates the HPBCD's inability to solubilize and form complex with Pal-L1 (Figure S17).

2.9 | Investigation of Intermolecular Cyclodextrin-Ligand Contacts with NOESY NMR

Subsequently, 2D NOESY NMR spectra between the different CDs and Pal-L1 were acquired to unravel complex's structural details. All the spectra were recorded at 298 K temperature. Initially, a 2D NOESY of the CDs (1 mM) in solution were acquired using a mixing time of 300 ms. Consequently, the complex with the ligand was acquired after the last point of the ^1H -NMR titration, at a 1:2 CD/Pal-L1 ratio. For the bound form, two trNOESY were acquired using two different mixing times, 150 and 300 ms, to detect spin-diffusion.

For the Pal-L1+RAMEA complex, several intermolecular NOEs were clearly observed. The protons located at the inner hydrophobic part of the cyclodextrin such as H3, H5, and even H6 presented clear NOE cross-peaks with the aliphatic saturated chain (CH_{2n}) of the palmitic acid scaffold of the lipopeptide. The randomly methylated groups of 2-, 3-, and 6-OMe methyl groups also displayed intense cross-peaks with the CH_{2n} of the palmitic acid, which is overlapped with γCH_2 of the Lys moiety. Additional cross-peaks between H3, H5, and H6 RAMEA and the CH_3 end of the palmitoylated tail were also observed. However, this terminal methyl group did not present NOEs with the OMe groups of the CD, which suggests that the palmitoylated tail fits within the CD ring in one unique direction (Figure 7).

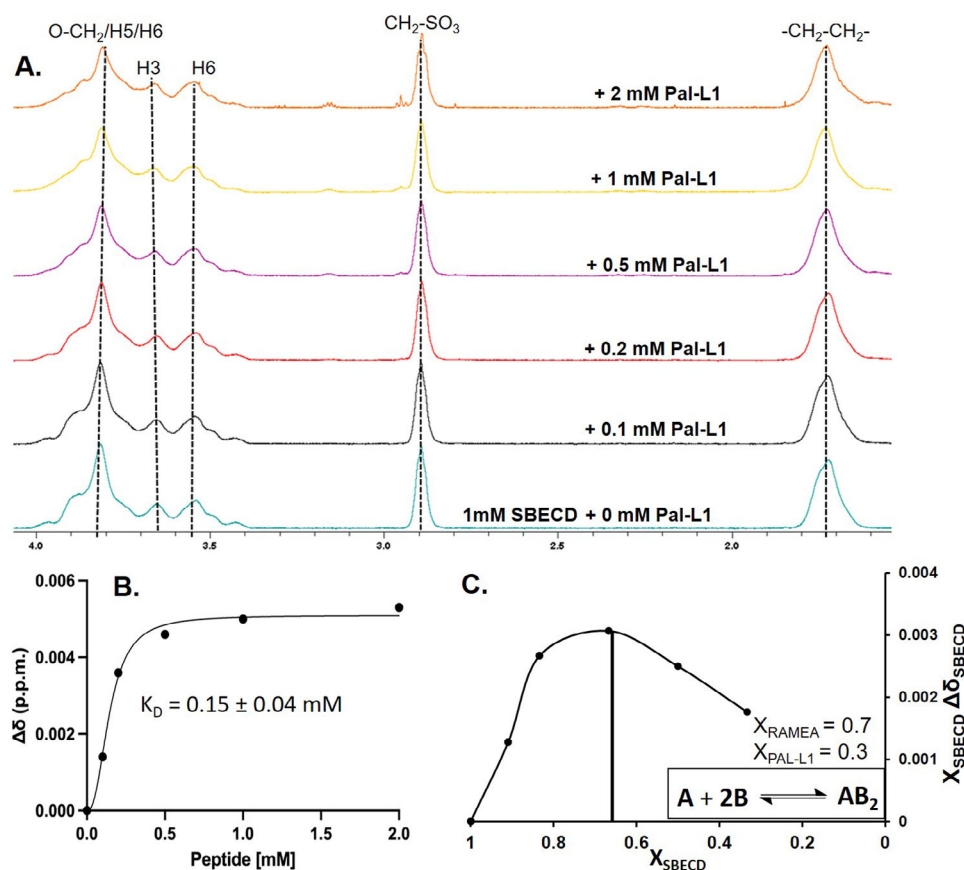


FIGURE 6 | ^1H -NMR titration between SBECD (1 mM) and Pal-L1. Some peaks of SBECD are selectively shifted upon increasing concentrations of Pal-L1 (A). The chemical shift perturbation in ppm versus peptide concentration (in mM) is plotted and fitted (B), and dissociation constant (K_D) obtained is calculated. Job's plot of the displaced ^1H resonances of SBECD throughout complexation with Pal-L1 is on panel (C).

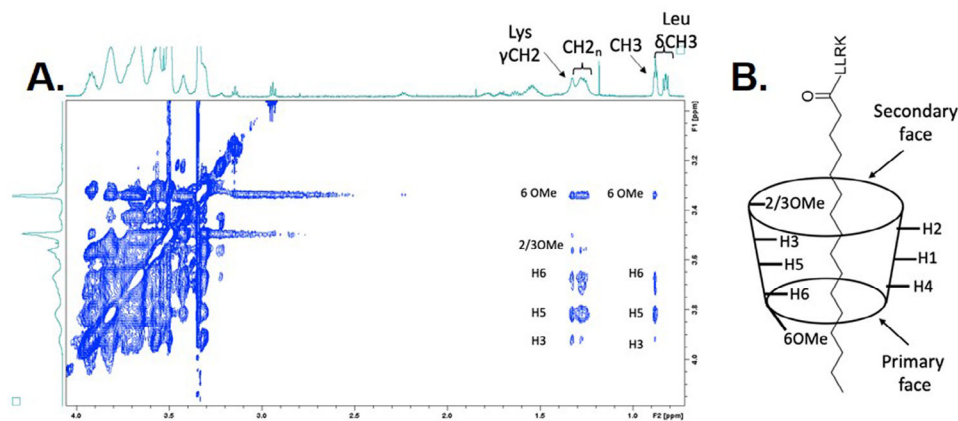


FIGURE 7 | 2D NOESY NMR of the complex between RAMEA and Pal-L1 (A). Schematic model illustrating the putative complex generated upon binding (B).

Regarding the trNOESY between the negatively charged SBECD and Pal-L1, several intermolecular NOEs were also observed, including those between H3, H5, and H6 at the hydrophobic core of the CD and the saturated chain CH_{2n} of the palmitic acid (Figure 8). Additional NOE-s were also observed between the CD protons and protons of the peptide residues. To differentiate the intramolecular NOE signals of SBECD from those between

the CD and the peptide (intermolecular), a NOESY spectrum of the CD alone was also acquired. Within those resonances, an intramolecular NOE cross-peak between the $n\text{Bu } \alpha\text{CH}_2$ (3.9 ppm) and the $n\text{Bu } \beta/\gamma\text{CH}_2$ (1.7 ppm) could be identified (Figure 8, in red). Then, when the NOESY of the complex was registered, more cross-peaks emerged between the CD protons and Arg $\beta/\gamma\text{CH}_2$, Lys $\beta/\delta\text{CH}_2$, and Leu $\beta/\gamma\text{CH}_2$ of the lipopeptide.

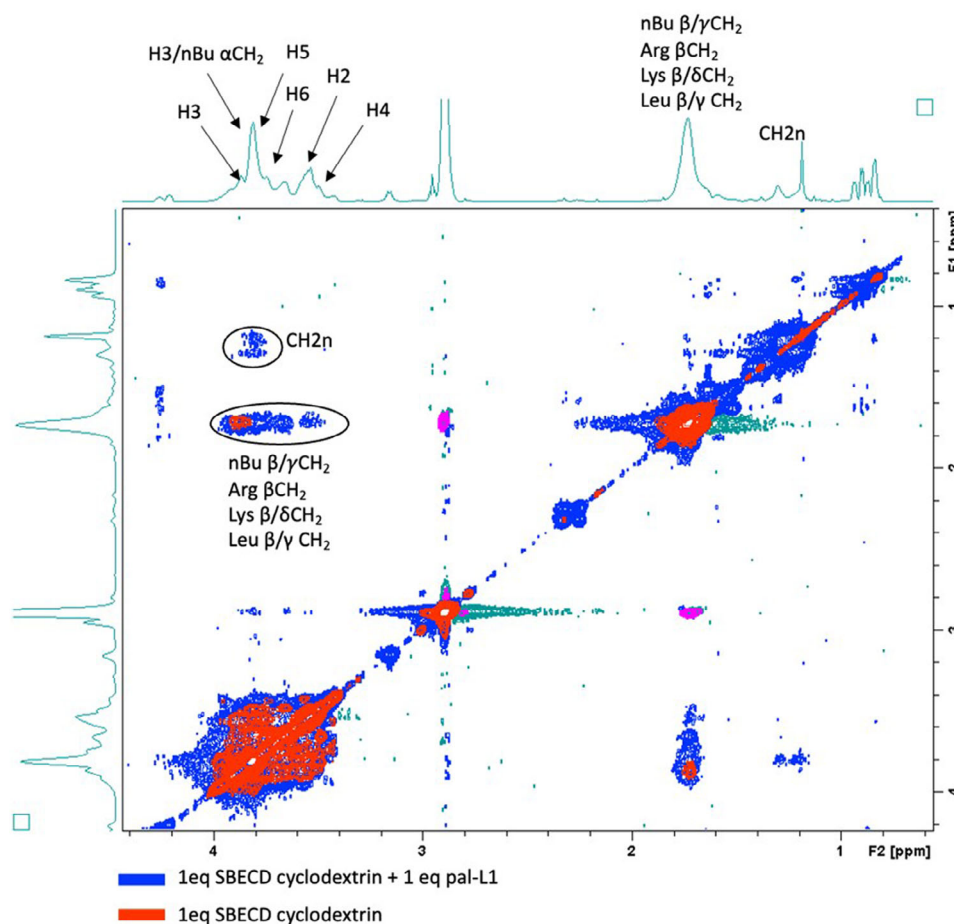


FIGURE 8 | 2D NOESY of SBECd free in solution (red) and bound to Pal-L1 (blue). Key intermolecular NOEs are indicated.

These intermolecular NOE cross-peaks suggest the presence of additional contacts between the lipopeptide and the SBECd, compared with the RAMEA.

2.10 | Molecular Dynamics Simulations

To better understand the structure of the complex formed between RAMEA and SBECd with Pal-L1, MD simulations were run. First, Pal-L1 was manually docked within the hydrophobic patch of the CDs, following the NOESY NMR observations, where H3, H5, and H6 of both CDs presented clear NOEs with the aliphatic chain of the palmitoyl group. The aliphatic chain of the palmitic acid was docked within the cyclodextrin, from top to bottom, from the wider part (secondary face) to the narrower site (primary face) of the cone-shaped CD. Both CD and lipopeptide molecules were parametrized using the general AMBER Force Field 2 (GAFF2) parameters, and 100 ns simulation run time was carried out with each complex using the standard procedures described in the experimental section.

The complex between RAMEA and Pal-L1 was kept stable throughout the entire MD simulation. The saturated chain of the palmitic acid was accommodated within the hydrophobic inner hole of the CD, establishing extensive contacts with H3, H5, and H6 of RAMEA, and also with the different randomly O-methylated groups (Figure 9). As also deduced from the NOESY

studies, the CH₃ end of the palmitic acid did not present NOEs with the 2- and 3-OMe of RAMEA, as they are far apart. Besides the hydrophobic contacts, charge-assisted H-bonds were established between the positively charged arginine and lysine residues with the 2-OH and 3-OH groups of the CD. Moreover, the backbone amide of the two Leu residues regularly established H-bonds with the 2- and 3-OMe groups of RAMEA. As a result, apart from the hydrophobic contacts between the hydrocarbon chain of the palmitic acid and the hydrophobic CD core, the charged lysine and arginine amino acids side chain probably contributes to anchoring Pal-L1 to RAMEA, forming a stable complex (Figure 9).

Regarding SBECd, a very stable complex was formed with Pal-L1 too, keeping the palmitic acid hydrocarbon chain within the hydrophobic core of the CD during the entire MD simulation. Significantly more non-bonded interactions were established between the charged residues of the peptide and SBECd than with RAMEA. In this case, the positively charged side chains of lysine and arginine not only establish H-bonds with the 2- and 3-OH groups on the rim of SBECd, but also form ion-pair interactions with the negatively charged sulfonate group of the CD (Figure 9). These ion-pair interactions are kept constantly throughout the entire simulation, and probably cause that the peptide residues closer to the CD are bent and, as a consequence, establish interactions between the amide backbone of the two Leu amino acids and the terminal carboxylate of the peptide with the 2- and 3-OH groups of SBECd. To sum this evidence up,

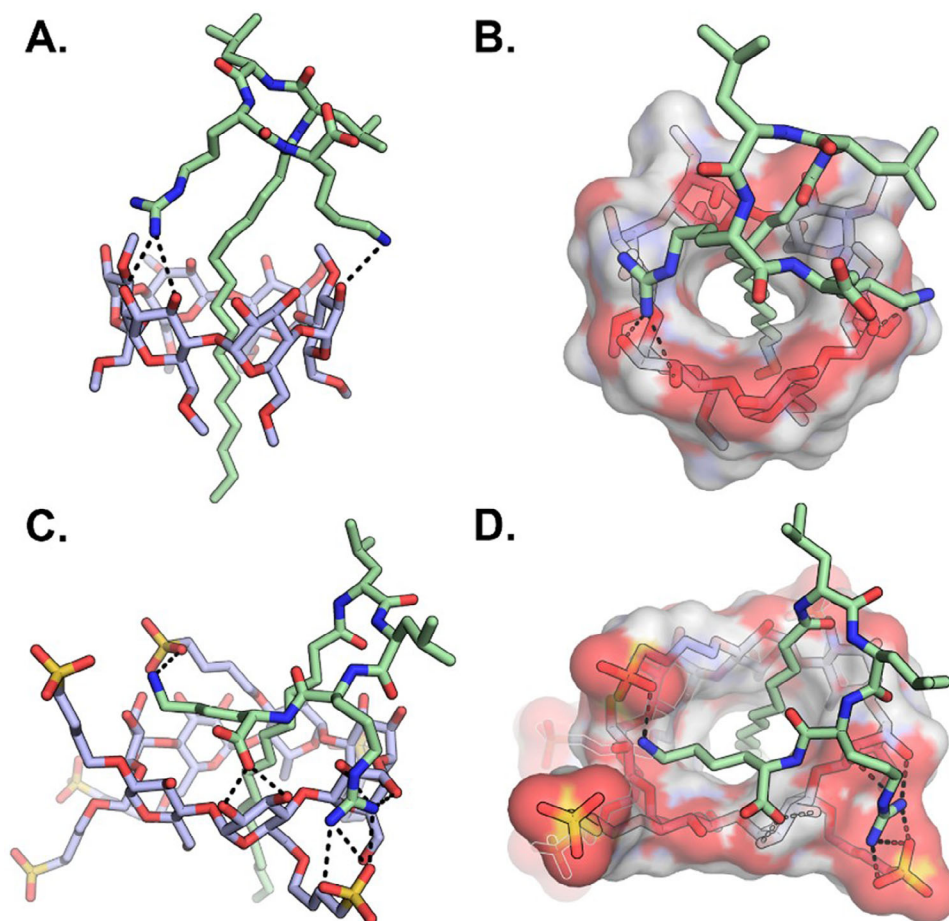


FIGURE 9 | Representative frames of the 100 ns MD simulation recorded for RAMEA (A and B) and SBECD (C and D) with Pal-L1. Complex between RAMEA (light blue C atoms) and Pal-L1 (green C atoms) (A). The Pal-L1+RAMEA complex is shown from above and RAMEA is represented with the solvent accessible surface (B). Complex between SBECD (light blue C atoms) and Pal-L1 (green C atoms) (C). The Pal-L1+SBECD complex is also shown from above, and SBECD is represented with the solvent accessible surface (D).

when forming a complex with SBECD, Pal-L1 engages in identical interactions as with RAMEA. Additionally, it may generate ion pair interactions with the sulfonates, further enhancing the stability of the complex.

Besides, the binding free energy (ΔG) and the energy components of the intermolecular interactions were also calculated by using Molecular Mechanics Poisson–Boltzmann Surface Area (MM-PBSA) analysis as implemented in AmberTools (see Supporting Information S8). The total binding energy between RAMEA and Pal-L1 was $\Delta G = -5.2$ kcal/mol, while for SBECD it was $\Delta G = -14.1$ kcal/mol. The results obtained suggest that Pal-L1 has a higher affinity for SBECD than for RAMEA and these results are in good agreement with NMR data.

2.11 | Structure–Activity Relationships of the Host–Guest Inclusion Complexes of Pal-L1+CDs

To improve the therapeutic applicability of Pal-L1, cyclodextrins were applied, and of those tested, RAMEA and SBECD were found to be the best candidates as they could improve solubility and reduce cytotoxicity.

It was shown that the palmitic chain of Pal-L1 was docked within the cavity of RAMEA with a stoichiometry of 1:1 and the dissociation constant (K_D) was 0.42 mM, while the association constant (K_A) was 2381 M^{-1} (Figure 5). The negatively charged SBECD interacts not only with the fatty acid chain but also with the positively charged side chain of the LLRK peptide, resulting in a lower K_D value, that is, 0.15 mM ($K_A = 6667 M^{-1}$) (Figure 6). By diffusion-ordered spectroscopy (DOSY) measurements, for Pal-L1+RAMEA complex, 2.36×10^{-6} m^2/s diffusion coefficient (D) was determined, and that value accounts for a size of 1.04 nm (Figure S18). For SBECD, similar observation was evidenced, having $D = 2.12 \times 10^{-6}$ m^2/s (1.16 nm) (Figure S17). These results are fully consistent with the average particle size of around 1 nm, measured by DLS (Figure 1H,I). Thus, we can conclude that Pal-L1 forms an ultralow-sized molecular dispersion system with CDs.

The formation of the host–guest inclusion complex and the molecular dispersion effect of CDs resulted in a cytoprotective activity, as the toxicity of Pal-L1, measured on different human cells was significantly reduced by the addition of RAMEA and SBECD (Figure 2). Reviewing the literature, the application of cyclodextrins to formulate fatty acid derivatives often results in

improved bioavailability, enabling the production of drugs and diagnostic reagents having enhanced efficacy [45].

In our study, three different cell types were applied of which HT29 cell line is often used as a model to study adsorption, transport, and secretion by intestinal cells [53], while HepG2 cells are widely exploited for investigating liver metabolism and hepatotoxicity [54]. The MonoMac-6 (MM6) cell line has been defined as a model system for studying monocyte function and biology and explored for drug toxicity toward human blood cells [40]. Interestingly, in all three cells, together with human red blood cells, the effect of Pal-L1 was very similar, having IC_{50} and HC_{50} values in the range of 10–20 μ M, and this membrane disrupting effect was abolished by the cyclodextrins in all cases (Figure 2). This suggests that the key element of the membrane perforating action of the Pal-L1 is the insertion of the fatty acid chain into the lipid bilayer, and the membrane binding affinity of Pal-L1 is highly comparable for all tested mammalian cells. The data indicate that the encapsulation of the fatty acid chain within the cyclodextrin cavity hinders its insertion into the cell membrane.

On the contrary, when the lipopeptide was tested on bacteria in the presence of cyclodextrin, similar MIC values were obtained as for the free peptide, so the antibacterial effect was preserved even in the complexed form (Figure 3). This implies that the molecular mechanism of the antibacterial action of the peptide is a bit different from the perforation, observed on the membrane of mammalian cells, and the positively charged amino acid side chains play a crucial role in the antimicrobial effect. This is also supported by the experimental observation that the negatively charged SBECD, which also interacts with the peptide side chains, slightly reduces the antibacterial effect, whereas this effect is rather increased in the case of the neutral RAMEA. Results of the liposome-based assays confirm this assumption, so it is likely that the interaction of the cationic Pal-L1 peptide with the anionic cytoplasmic membrane of the Gram-positive bacteria is promoting the membrane binding and consequently the pore formation.

Due to the cytoprotective effect on mammalian cells, the selectivity index of the Pal-L1 peptide was greatly enhanced by the cyclodextrins. To study this in more detail, we performed a titration experiment in order to establish the optimal peptide/CD ratios at which both the cytoprotection of CDs and the antimicrobial effect of the Pal-L1 peptide are manifested (Table 4).

It is important to note that CD complexation is a dynamic process, so that association and dissociation between the host-guest partners constantly occur and the estimated lifetime of the complex is in the range of milliseconds or even less [55]. The strength of the complexation is characterized by the association constant (K_A), while the ligand release is defined by the dissociation constant (K_D), which is reciprocal of K_A . Equilibrium states can be assigned with the formula of $K_D = [L][CD]/[LCD]$, where [L] is the concentration of the free ligand, [CD] is the concentration of the free CD, and [LCD] is the concentration of the inclusion complex, in equilibrium. Dissociation due to dilution is the major release mechanism in the case of ligand-CD complexes, and competitive processes such

as membrane binding, cellular uptake, or ligand displacement can shift the equilibrium and contribute to the increased ligand release.

Based on the K_D values, we can calculate the amount of complexed and free form of the Pal-L1 peptide in equilibrium for a given peptide/CD mixing ratio and see that these data are well correlated with cell viability values. Figure 10 demonstrates the graphical representation of the experimental results showing the amount of free and complexed form of Pal-L1 in equilibrium, together with the hemolytic and antibacterial activity. From the graph, we can define a selectivity window, that is, if RAMEA is applied at a ratio of at least 1/10, the cytoprotective effect is present. For SBECD, this ratio is only 5 equivalents.

We assumed that the higher binding affinity of the Pal-L1 peptide to the bacterial membrane having larger magnitude of negative electric charge results in a shift in the equilibrium toward Pal-L1 release, while in the case of mammalian cells, the membrane affinity and the complex dissociation are probably at a comparable level, so the membrane binding does not alter that much the equilibrium state.

3 | Conclusions

As antimicrobial resistance becomes an increasingly prevalent challenge, increasing attention has been placed on AMPs as a promising alternative to classical antibiotics. USCLs are a synthetic class of AMPs characterized by a fatty acid moiety, attached to short cationic peptide. They offer promising features, such as a facile, economic, rapid synthesis, and low susceptibility of developing resistance. However, their potential shortcomings, including poor solubility in aqueous solutions and a lack of selectivity, negatively impact their therapeutic use.

In our study, we prepared a panel of USCLs from a short cationic tetrapeptide (L1) derived from the C-terminal active site of a well-known AMP, buforin II. We observed that above C10 length, fatty acid coupling confers a significant antibacterial effect to the otherwise inactive small peptide. On the other hand, these lipopeptides showed significant cytotoxic and hemolytic side effects. Note, that the IC_{50} and HC_{50} values correlated well with the length of the fatty acid chain (Table 2), suggesting that the fatty acid insertion is the key element of membrane disruption and pore formation. Of the lipopeptides, Pal-L1 was selected for further studies as it was non-selective and forms aggregates in the micrometer range in physiological media (Figure 1B,C), which makes it an exciting model to study the typical challenges of this group of molecules.

We investigated six different cyclodextrins as a formulation for Pal-L1 to limit its drawbacks. Our results proved that the α -cyclodextrin derivative RAMEA and the β -cyclodextrin derivative SBECD were suitable agents to improve the solubility and form host-guest inclusion complexes with the Pal-L1 lipopeptide. Interestingly, the two cyclodextrins form complexes with the lipopeptide with different structures and stoichiometries and the dissociation constants are consequently also distinct. Electrostatic attraction between negatively charged functional groups on

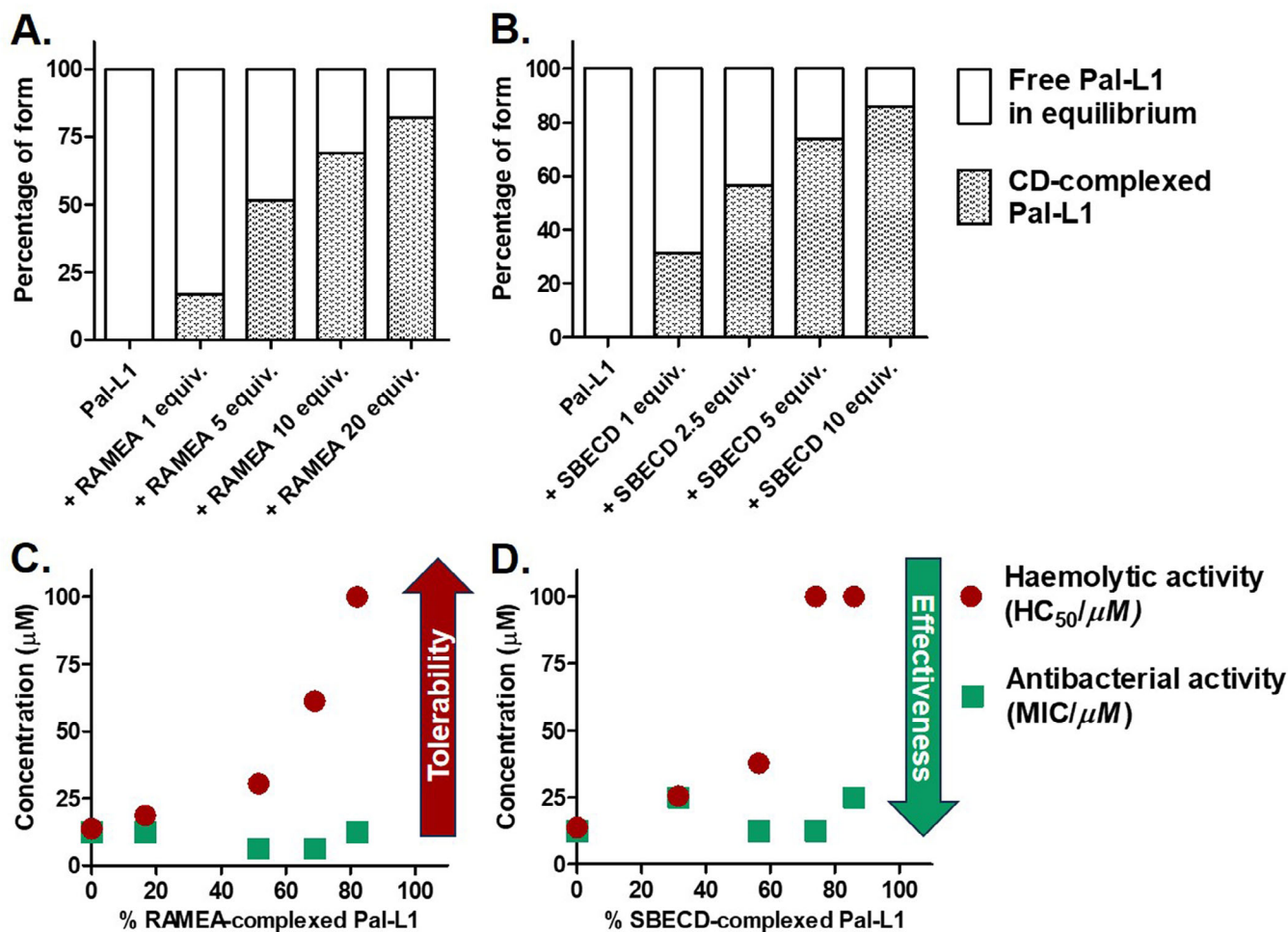


FIGURE 10 | Correlation between the concentration of the CD-complexed Pal-L1 and the biological activity. Left panel shows the results with RAMEA (A and C), while on right panels the results obtained with SBECD are presented (B and D). The equilibrium concentration values for the free and CD-complexed peptide were calculated based on the K_D values, obtained from the $^1\text{H-NMR}$ titration experiments (A and B). Red dots represent the half-maximal hemolytic concentration (HC_{50}) of the Pal-L1 peptide in the presence or absence of CDs, determined by the hemolysis assays. Green squares correspond to the minimal inhibitory concentration (MIC) values, measured against MRSA bacteria (C and D).

the SBECD cyclodextrin and the positively charged side chains on the peptide ensures enhanced host-guest affinity in addition to the hydrophobic interaction.

The formation of the inclusion complex and the aggregation destructing effect of cyclodextrins resulted in a cytoprotective activity, as the toxicity of Pal-L1, measured on different human cells, was significantly reduced by the addition of RAMEA and SBECD. In contrast, the antibacterial effect of the lipopeptide was not altered by the cyclodextrins. By using 10 equiv. of RAMEA and 5 equiv. of SBECD, the selectivity index of Pal-L1 can be greatly improved.

This phenomenon can be attributed to differences in the composition and charge of the mammalian cell membrane and the bacterial cell wall. The positively charged peptide binds with higher affinity to membranes enriched in anionic components, and given the high amount of negatively charged lipoteichoic acid present in the cell wall of Gram-positive bacteria, it is assumed that this is the reason for the selectivity. It is likely that the equilibrium states are shifted by this preferential electrostatic

interaction, hence the bacterial cell wall is favored by the peptide over the mammalian cell membrane (Figure 11). The experimental results obtained will definitely be worth complementing with MD simulations, which we plan to do in the future.

In addition, the role of leucine side chains will be worthwhile to investigate and to study changes in membrane activity by systematic amino acid substitutions in this USCL panel.

As supramolecular complexes of lipopeptides show encouraging results in the *in vitro* biosystems, their further examination is of interest. Investigating the *in vivo* behavior, plasma stability, biodistribution, and antibacterial activity of the host-guest complexes may be particularly relevant. It is worth further investigating whether and to what extent resistance to these complexes develops. Overall, water-soluble, freeze-dried, reconstituted, and sterile-filtered lipopeptides in their cyclodextrin complexes may represent a new class of antimicrobial agents that can help combatting infectious diseases, either alone or in combination with other antibiotics.

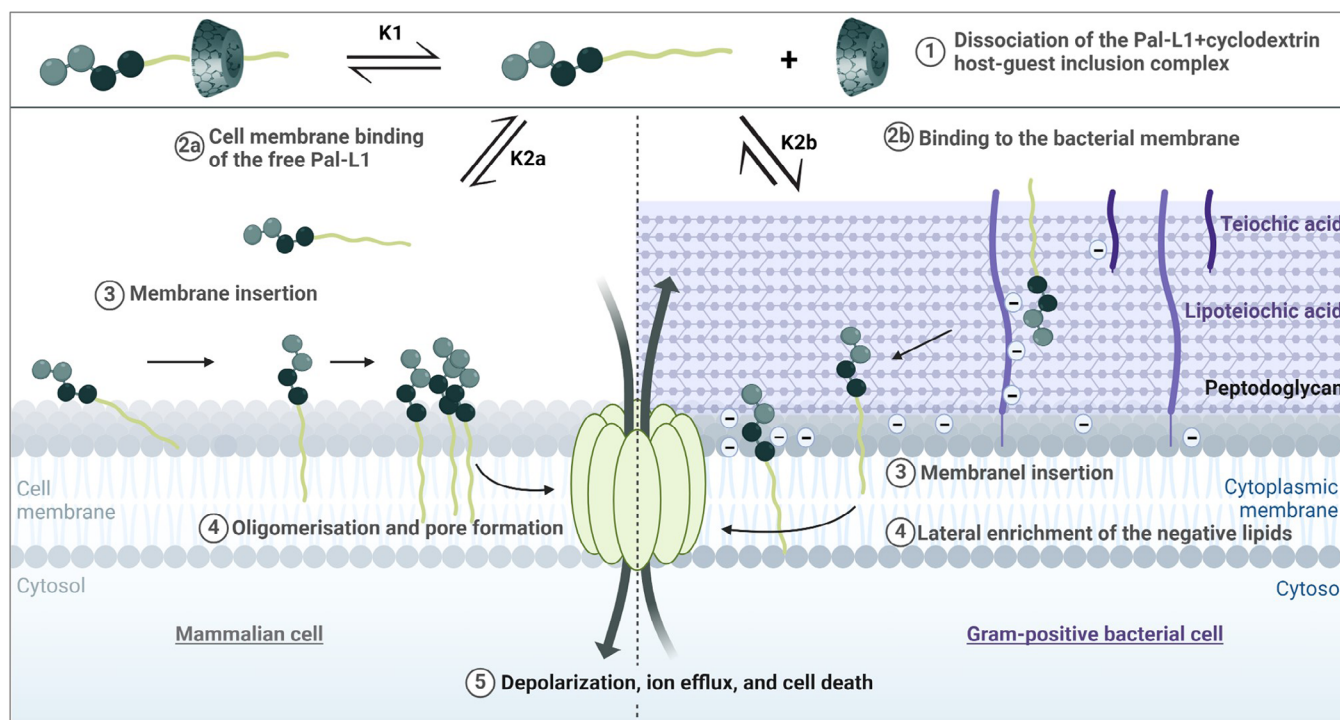


FIGURE 11 | Difference between mammalian cell membranes and Gram-positive bacterial cell wall and the putative mechanism of pore formation. It is assumed that the positively charged Pal-L1 lipopeptide binds to the more negatively charged membranes of bacteria with greater affinity compared to the mammalian cell membranes. When the peptide encounters mammalian cells, it prefers the complexed state with cyclodextrin, because membrane binding does not give enough energy gain, and the membrane bound state is likely energetically less favorable. In contrast, a stronger affinity for the more negatively charged bacterial membrane shifts the balance toward the dissociated form and the Pal-L1 lipopeptide prefers to reside in the lipid bilayer rather than buried in cyclodextrin. We speculate that K_1 is falling in the range of K_{2a} , while K_{2b} is markedly higher.

4 | Experimental Section

4.1 | Materials

Amino acid derivatives and piperidine were purchased from Iris Biotech (Marktredwitz, Germany). *N,N'*-diisopropylcarbodiimide (DIC), 1-hydroxybenzotriazole (HOBt), 1,8-diazabicycloundec-7-ene (DBU), hydrazine monohydrate ($N_2H_4 \cdot H_2O$), triisopropylsilane (TIS), palmitic acid, valeric acid, decanoic acid, myristic acid, stearic acid, arachidic acid, behenic acid, Fmoc-Rink Amide MBHA resin, and Resazurin sodium salt were obtained from Merck (Budapest, Hungary). Trifluoroacetic acid (TFA), *N,N*-dimethylformamide (DMF), dichloromethane (DCM), diethyl ether, and acetonitrile (AcN) were purchased from VWR (Budapest, Hungary). RPMI-1640, PBS, 2 mM L-glutamine were from Lonza (Basel, Switzerland), while trypsin, non-essential amino acids, and penicillin and streptomycin were purchased from Gibco (Thermo Fisher Scientific, Waltham, MA, USA). Fetal bovine serum (FBS) was from EuroClone (Pero, Italy). Sulfobutylether- β -cyclodextrin sodium salt (SBECD) (Degree of Substitution $DS \sim 6.5$), (2-hydroxypropyl)- β -cyclodextrin (HPBCD) ($DS \sim 4.5$), (2-hydroxypropyl)- γ -cyclodextrin (HPGCD) ($DS \sim 4.5$), randomly substituted methyl- α -cyclodextrin (RAMEA) ($DS \sim 11$), randomly substituted methyl- β -cyclodextrin (RAMEB) ($DS \sim 11$), randomly substituted methyl- γ -cyclodextrin (RAMEG) ($DS \sim 11$) were provided by CycloLab Cyclodextrin Research and Development Ltd. (Budapest, Hungary). High purity synthetic 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-*sn*-glycero-3-

[phospho-*rac*-(1-glycerol)] (sodium salt) (DOPG) were purchased from Avanti Polar Lipids Inc. (USA). Analytical reagent grade chloroform (LabScan, Hungary) and methanol (Reanal, Hungary) were used for lipids solutions.

4.2 | Peptide Synthesis

L1 (Leu-Leu-Arg-Lys) was synthesized manually on solid phase (Fmoc-Rink Amide MBHA resin, capacity = 0.51 mmol/g) by Fmoc/^tBu strategy with DIC/HOBt coupling reagents. To obtain the USCLs panel, saturated fatty acids were coupled at the *N*-terminus of L1 using the same coupling method.

Peptides were cleaved in TFA using $H_2O:TIS = 3:3$ (% v/v) as scavengers (3 h, RT). After filtration, crude products were precipitated in cold diethyl ether, centrifuged (4000 rpm, 5 min), and freeze-dried. Crude peptides were purified by RP-HPLC on a semipreparative Phenomenex Jupiter Proteo C12 column (10 μm , 90 $\text{\AA}$, 10 $\times$ 250 mm) with linear gradient elution using 0.1% TFA in water (Eluent A) and 0.1% TFA in AcN:water = 80:20 (% v/v) (Eluent B) on an UltiMate 3000 Semiprep HPLC (Thermo Fisher Scientific, Waltham, MA, USA). Purified products were characterized by RP-HPLC (LC-40 HPLC System from Shimadzu, Kyoto, Japan) on an analytical C12 column using gradient elution with the eluents A and B (flow rate was 1 mL/min, the gradient was 5–100 B% in 20 min, unless otherwise specified in the text, UV detection at $\lambda = 220$ nm). The molecular mass ($M_{meas.}$) was determined using a Thermo Scientific (Waltham, MA, USA)

QExactive Focus Hybrid Quadrupole-Orbitrap Mass Spectrometer. The instrument parameters were set as follows: capillary voltage: 3.5 kV, flow rate: 0.300 mL/min, heated capillary temperature: 256.25°C.

For the prediction of the different physical/chemical properties of the lipopeptides, calculator plugins of the Chemicalize program (<https://chemicalize.com/app/calculation>) was used.

4.3 | Antibacterial Activity

Antibacterial activity of the USCLs was determined on well-characterized strains of major human pathogenic bacteria, namely, the Gram-positive *S. aureus* MSSA (ATCC 29213) and *S. aureus* MRSA (ATCC 33591), and on the Gram-negative *E. coli* (ATCC 25922) and *P. aeruginosa* (ATCC 27853) using the broth microdilution method in Mueller Hinton Broth (MH, Biolab, Budapest, Hungary) [56]. Fresh overnight cultures of MSSA, MRSA, *E. coli*, and *P. aeruginosa* were used to prepare 0.5 McFarland suspensions in 0.9% NaCl. This was further diluted 1:100 in MH broth, and 50 µL was aliquoted to each well on a 96-well round bottom plate (Sarstedt). Growth control (without the antimicrobial compounds) and sterile control (no bacteria, no drug) were also applied in eight parallels on each microtiter plate.

USCLs were dissolved at 20 mM concentration in DMSO and then diluted 100 times with MH broth, immediately before the experiment. Ten-step, two-fold dilution series of the compounds were prepared in MH broth and 50 µL of each dilution was added to the bacterial suspension. The final concentration of bacteria was 5×10^5 cfu/mL, and the tested concentration range for the peptides was 100–0.2 µM. Plates were incubated in ambient air for 18–20 h at 35°C. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of the compound at which no visible growth of the bacterium occurred. In addition, optical density was also determined at 595 nm, using a Thermo Scientific Multiscan FC plate reader. All measurements were performed in duplicates, and the mean bacterial density, relative to the untreated control wells, was graphically presented, together with the standard deviation (SD).

For internal QC, susceptibility testing of the ATCC control strains was performed to several antibiotics (vancomycin, gentamicin, norfloxacin, and ciprofloxacin), and the obtained MIC values were always within the expected range as indicated by the EUCAST guidelines (the European Committee on Antimicrobial Susceptibility Testing; routine and extended internal quality control for MIC determination and disk diffusion as recommended by EUCAST, Version 14.0, 2024, available online at https://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/QC/v_14.0_EUCAST_QC_tables_routine_and_extended_QC.pdf).

4.4 | Solubility Studies on Pal-L1 by RP-HPLC

Two microliters of a 20 mM DMSO stock solution of Pal-L1 was diluted 1:200 with eluent B, spun down, and 100 µL of the sample was analyzed by RP-HPLC in a C12 analytical column using gradient elution with the eluents A and B (flow rate: 1 mL/min,

gradient: 40–100 B% in 10 min, UV detection at $\lambda = 220$ nm). The retention time (RT) and the AUC were extrapolated from the chromatogram.

A sample prepared using the same procedure was spun down using a nylon centrifugal filter (cutoff: 0.2 µm), and 100 µL of the filtrate was analyzed by RP-HPLC as mentioned above. The RT and the AUC were then compared to the corresponding Pal-L1 non-filtered sample to ensure the suitability of the validation method.

Then a calibration curve was built with standard peptide concentrations (ranging from 25 to 150 µM) to establish the linear correlation between peptide concentration and AUC values. The linear regression equation and the coefficient of determination (R^2) were obtained from the curve.

The same analytical protocol was used to determine the solubilization efficacy of the cyclodextrins. Briefly, 2 µL of the DMSO peptide stock solution was dissolved in 198 µL of aqueous CD solution (100 mM), followed by centrifugation using a nylon centrifugal filter, and then 100 µL of the filtrate was further diluted 1:1 (% v/v) with eluent B and analyzed by RP-HPLC. The AUC values obtained per sample were compared to the AUC of the internal standard control (Pal-L1 dissolved in eluent B) and expressed as AUC%. Pal-L1 dissolved in water was used as a negative control.

4.5 | Pal-L1 Solubility Studies Mimicking the Cell Studies Using RP-HPLC

Two microliters of the Pal-L1 DMSO stock solution (20 mM) was dissolved in 198 µL of RPMI-1640, then the solution was spun down using a nylon centrifugal filter, and 100 µL of the filtrate was further diluted 1:1 with eluent B and analyzed by RP-HPLC as described above.

Then, 2 µL of the Pal-L1 DMSO stock solution (20 mM) was dissolved in 38 µL of aqueous CD solution (50 mM), and then the solution was diluted with 160 µL of RPMI-1640. The same procedure was followed to analyze the sample.

4.6 | Cells

MonoMac-6 (MM6) human monocytic cell line (Cellosaurus ID: CVCL_1426), HT29 human colon adenocarcinoma cells (CVCL_0320), and HepG2 human hepatocellular carcinoma cells (CVCL_0027) were maintained as an adherent culture in RPMI-1640 supplemented with 10% FBS and with 2 mM L-glutamine, 1% non-essential amino acids, 1% penicillin-streptomycin (from 10,000 units of penicillin and 10 mg of streptomycin/mL) at 37°C in a humidified atmosphere containing 5% CO₂.

4.7 | Cytotoxicity

Cytotoxicity was investigated using AlamarBlue viability assay. Cells were seeded a day prior to the experiment in a 96-well tissue culture plate (10,000 cells/well). The day after, the cells were

treated with the peptides (up to 100 μM) in serum-free media for 24 h. After centrifugation (1000 rpm, 5 min) and washing with serum-free medium, 22 μL of AlamarBlue reagent (resazurin sodium salt was dissolved in PBS at 0.15 mg/mL and pH 7.4, and then sterilized, filtered prior use) was added. After 3 h of incubation, the fluorescence was detected ($\lambda_{\text{Ex}} = 530/30$ and $\lambda_{\text{Em}} = 610/10$ nm) in a Synergy H4 multimode microplate reader (BioTek, Winooski, VT).

Cytotoxicity of CDs and Pal-L1 formulated with the different CDs was investigated using the same procedure, after sample preparation. In brief, the peptide was solubilized in DMSO (20 mM), and then the solution was added to a 50 mM aqueous CD solution to obtain a 200 μM peptide concentration (final DMSO concentration 5% v/v). The sample was further diluted in ICM media prior to incubation (peptide concentration up to 100 μM , CD concentration up to 5 mM). All measurements were performed in quadruplicate, and the viability of treated cells was compared to the untreated control (relative viability %). All data are presented as mean $\pm$ SEM. The 50% inhibitory concentration (IC_{50}) values were calculated from the dose-response curves after fitting with non-linear regression. The selectivity index (SI) for *S. aureus* strains versus MM6 was defined as the $\text{IC}_{50}/\text{MIC}$ ratio.

4.8 | Hemolysis Assay

The hemolytic activity of the compounds was tested on human red blood cells (RBC). RBC concentrate from healthy volunteers was obtained from the Hungarian National Blood Transfusion Service. First, RBC was washed twice with PBS, then diluted with PBS (2 v/v RBC%) and plated on a 96-well, round-bottom plate at 50 μL volume. Cyclodextrins and Pal-L1 peptide were solubilized as previously described. The highest cyclodextrin concentration was 5 mM, while the highest Pal-L1 concentration was 100 μM . The final RBC concentrate was 1% v/v and the final volume was 100 μL in the wells. Plates were incubated at 37°C, 5% CO_2 for 2 h, then centrifuged (2000 rpm, 5 min, 4°C), and 50 μL of the supernatants was carefully transferred to a new plate, containing 100 μL H_2O in each well. Optical density (OD) was measured at 414 and 450 nm by a Synergy H4 multimode microplate reader (BioTek, Winooski, VT). The percentage of hemolysis was compared to the positive control bee venom Melittin. All data were presented as the mean $\pm$ SD ($N = 4$).

4.9 | Complex Stability in Solution and After Freeze-Drying Using RP-HPLC

Pal-L1 (20 mM DMSO stock solution) was dissolved in 198 μL of aqueous RAMEA or SBECD solution (10 mM). The sample was processed as described above, and 100 μL was analyzed by RP-HPLC at Time 0. The remaining solution was kept at RT for 24 h and then analyzed by RP-HPLC. The comparison of the AUCs at Time 0 and at 24 h was used as an index of the chemical stability of the complex in solution.

The stability of the complex through the freeze-drying process was obtained by comparing the AUCs of the Pal-L1 formulated by CDs before and after lyophilization. Briefly, the samples were prepared as mentioned above. Then 100 μL was analyzed at Time

0 by RP-HPLC, the remaining 100 μL was freeze-dried and then resuspended in the same volume of water, injected and analyzed by RP-HPLC.

4.10 | Liposome Preparation

For mimicking the mammalian cell membranes, unilamellar, pure DOPC (PC) was used, while for the bacterial model DOPC/DOPG (PCPG) at a molar ratio of 8:2 was applied. Liposomes were prepared by the lipid thin film hydration technique. PC and PCPG were dissolved in chloroform containing 50% v/v methanol. After solvents evaporation by a rotary evaporator, the resulting lipid films were kept in vacuum overnight to remove the residual traces of the organic solvent. The dry lipid films were then hydrated using a 10 mM, pH = 7.4 PBS solution (Sigma-Aldrich, Hungary) to a final lipid concentration of 10 mg/mL. The samples were homogenized thorough freeze-thaw cycles (10 times), using liquid nitrogen (-196°C) and water bath ($\sim 37^\circ\text{C}$) and then were extruded (at least 10 times) through polycarbonate filters (Nuclepore, Whatman Inc.) with 100 nm pore size using a LIPEX extruder (Northern Lipids Inc., Canada).

4.11 | Dynamic Light Scattering

Particle size and size distribution of the samples were measured using W130i DLS apparatus (AvidNano, UK). Low volume disposable cuvettes with 1 cm path-length were used (UVette, Eppendorf Austria GmbH). Samples containing 50 μM peptide and 635 μM liposomes were measured in a final volume of 80 μL in MilliQ water. The time-dependent autocorrelation functions were measured for 10 s and repeated 10 times. The analysis of the measurement data was performed with the i-Size software, supplied with the device.

4.12 | NMR

The NMR experiments were recorded in Bruker AVANCE 2 600 MHz spectrometer, equipped with a standard triple-channel probe and/or in a Bruker 800 MHz spectrometer, equipped with a TCI cryo-probe (Bruker, Billerica, MA, USA).

The total volume for the NMR samples was 500 μL and 5 mm precision NMR tubes were used (New Era Enterprises, Vineland, USA). The pH of the samples was monitored with pH meter Crison Basic 20 (Crison Instruments SA, Barcelona, Spain) and adjusted, if necessary, with NaOH and HCl or NaOD and DCl.

4.13 | ^1H -NMR Titrations

For obtaining the dissociation constant of the complexes between the lipopeptides and the different cyclodextrins (RAMEA, SBECD, RAMEB, and HPBCD) and determining the complex stoichiometry, ^1H -NMR titrations were carried out. For that purpose, 1 mM of the corresponding CD was dissolved in deuterated phosphate saline buffer (20 mM sodium phosphate, 150 mM NaCl, pD 7.4). Increasing equivalents of lipopeptide were added until a 1:2 CD/lipopeptide ratio was reached. The temperature

was set at 298 K. For the calculation of the K_D , $\Delta\delta H$ were plotted versus the Pal-L1 concentration and fitted according to a classical Langmuir isotherm. For the stoichiometry calculation, the classical Job's plot method of continuous variation was generated, as detailed elsewhere [52, 57].

4.14 | Molecular Dynamics Simulations

For the complexes formed between the Pal-L1 lipopeptide with the RAMEA and SBEC cyclodextrins, the acyl chain of the Pal-L1 was manually docked using PyMol software within the hydrophobic cavity of the CDs, considering the experimental data obtained by analysis of the key intermolecular NOEs assigned in the 2DNOESY NMR experiment. The general Amber force field (GAFF2) [58] was used for used for both CD and Pal-L1 molecules to carry out the MD simulations. The initial structures were neutralized with either Na^+ or Cl^- ions and set at the center of a cubic TIP3P64 water box, with a buffering distance between solute and box of 10 Å. For each system, a two-stage geometry optimization approach was followed: the first stage minimized only the positions of the solvent molecules and ions, while the second stage provided an unrestrained minimization of all the atoms in the simulation cell. The systems were then heated by incrementing the temperature from 0 to 300 K under a constant pressure of 1 atm and periodic boundary conditions. Harmonic restraints of 10 kcal/mol were applied to the solute under the Andersen temperature coupling scheme [59]. The time step was kept at 1 fs during the heating stages, allowing potential inhomogeneities to self-adjust. Water molecules were treated with the SHAKE [60] algorithm such that the angle between the hydrogen atoms was kept fixed through the simulations. Long-range electrostatic effects were modeled using the particle mesh Ewald (PME) method [61]. An 8 Å cutoff was applied to non-bonded interactions. Each system was equilibrated for 2 ns with a 2 fs time step at a constant volume and temperature of 300 K. For each system, three 100 ns simulations were recorded, accumulating a total MD time of 300 ns.

4.15 | Statistical Analysis

All values are expressed as mean $\pm$ SD. Data were normally distributed, and comparison between groups was performed by using one-way ANOVA, followed by Tukey's post hoc test. A value of $*p < 0.05$ was considered significant.

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Conflicts of Interest

The authors declare no conflicts of interest.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.