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Original Communication

Arginine-lysine swaps selectively enhance antimicrobial activity over cytotoxic activity of LL-37 peptide

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ABSTRACT

LL-37 is the only cationic peptide belonging to the cathelicidin family expressed in humans. LL-37 has bactericidal activity and exerts immunomodulatory functions, thus forming, together with other peptides, the first line of defense against infections. The formation of LL-37 aggregates in the presence of neutral membranes promotes lack of specificity for microbial cells, which could explain why LL-37 becomes cytotoxic towards eukaryotic cells at high concentrations. Cationic amino-acids such arginine (Arg) and lysine (Lys) are known determinants for bacterial killing; however very little is known about how Lys-Arg exchange can influence LL-37 biological activities. Since antimicrobial peptides are promising candidates for the development of novel anti-infective agents, we have compared the bactericidal and cytotoxic effects of five LL-37 variants with wild-type peptide. The bactericidal activity was tested against *Escherichia coli* and *Streptococcus agalactiae*, while cytotoxicity was measured against A549, a human bronchoepithelial cell line. We found clear differences in bacterial killing kinetics towards both pathogens when central Arg residues were mutated in Lys, with Arg more efficient than Lys in bacterial membrane permeation. Of interest, the Arg at position 34 can compensate for the absence of the Arg at position 19 and 23 and the presence of Lys at the other positions resulted in a diminished toxicity for eukaryotic cells. Our study sheds new light on key amino-acid residues of LL-37 and should be considered when novel cationic amphipathic peptides derived from LL-37 are designed.

KEYWORDS: antimicrobial, peptide, LL-37, cytotoxicity.

INTRODUCTION

The challenge posed by the rapid increase in antibiotic-resistant bacterial strains in the post-antibiotic era has raised the interest in searching for novel anti-infective agents with a different mode of action. Antimicrobial peptides (AMPs) are natural antibiotics that represent an intriguing starting point to develop new compounds against antibiotic-resistant pathogens. AMPs are key components of the innate immune system, widely distributed throughout all the kingdoms of life and among the most ancient host defense factors [1, 2]. Defensins and cathelicidins are the most representative AMP families. Defensins can be grouped in two subfamilies, α and β, but while different members of this family are widely expressed in different human tissues [3], LL-37 is the only cathelicidin found in humans [4]. LL-37 is released as active form by proteolytic cleavage of its precursor hCAP-18 at the C-terminal [5]. LL-37 stimulates angiogenesis, induces proliferation of lung epithelial cells, accelerates wound healing of the airway epithelium, cytokine release and cell migration [6] and contributes to inflammatory

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and tissue remodeling processes [7]. The LL-37 cationicity of +6 positive net charge, due to the presence of positively charged amino-acids, such as Arg and Lys residues, allows binding to bacterial membranes as well as to polyanionic macromolecules like DNA, RNA and filamentous (F)-actin. In cystic fibrosis patients, the binding of LL-37 to anionic molecules leads to a reduced antibacterial activity [8]. LL-37 complexed with self-DNA or self-RNA has also been found in skin lesions of psoriatic patients [9, 10] and in lupus erythematosus [11, 12], thus triggering the autoimmune response. These findings show the relevance of LL-37 for human health and explain why its biological properties and mode of action have been extensively studied in the last years. We have previously demonstrated that four out of the five Arg residues present in LL-37 could be targets of ADP-ribosylation, a post-translation modification, which, by the addition of the negatively charged ADP-ribose moiety, can alter the overall cationicity of the peptide [13]. In addition, we found that LL-37 forms stable aggregates and toroidal pores in the presence of negatively charged membranes. A more extended oligomerization is seen also in the presence of mammalian-like membranes thus leading to a reduced selectivity of LL-37, which preferentially binds to bacterial versus eukaryotic cells [14]. This finding shows that LL-37 structure and activity are deeply connected. In addition, recent observations showed that Arg-Lys swap could influence the biological properties of truncated forms of LL-37 [15]. Therefore, we investigated the effects of Arg to Lys mutations on the antimicrobial and cytotoxic activities of LL-37. Herein we aimed to identify critical effects on microbicidal and cytotoxic activities induced by multiple substitutions of Arg residues of LL-37. The antimicrobial activity was tested against *Escherichia coli* and *Streptococcus agalactiae*, while cytotoxicity was measured against the human bronchoepithelial cell line A549. We found that peptides with selected Arg-Lys swaps had an improved microbicidal activity and reduced cytotoxicity versus eukaryotic cells as compared to wild-type LL-37. Therefore, this discovery should be taken into consideration for the design of LL-37-derived therapeutic peptides.

MATERIALS AND METHODS

Reagents

SYTOX Green and Dulbecco's modified Eagle's medium (DMEM) were from Invitrogen, and 3-[4,5-dimethylthiazol- 2-yl]-2,5-diphenyltetrazolium bromide (MTT) was from Trevigen. All other reagents used in this study were from Sigma Aldrich.

Peptide synthesis

The synthetic peptide LL-37 and LL-37 analogs were synthesized at the Protein and Peptide Chemistry Facility of the University of Lausanne, Switzerland. Peptides were purified by high-performance liquid chromatography to more than 95% purity. Identity of peptides was confirmed by mass spectrometry.

Circular dichroism

Circular dichroism (CD) experiments were performed at room temperature on a Jasco CD-J-815 spectropolarimeter using a quartz cuvette with a path length of 1 mm. Wild-type and mutant LL-37 peptides were dissolved in 50 mM 3-(N-morpholino)propanesulfonic acid (MOPS) at pH 6.8 and were incubated with vesicular suspension ranging from 0.15 to 7.5 mM concentration. For experiments in the presence of the folding agent, 50% (v/v) TFE was added to 0.03 mM of wild-type LL-37 and mutants to check the capability of peptides to assume a defined α-helix conformation.

Each CD spectrum was recorded from 190 to 250 nm at room temperature on a JASCO-815 spectrometer and was accumulated at least ten times to improve the signal-to-noise ratio. Baselines of either solvent or membrane olution without peptide were subtracted from the respective sample spectra to calculate the peptide contribution. The quantitative analysis of the CD spectra was performed using the freely available program "DICHROWEB" (Birkback, London College) [16].

Liposome Preparation

Liposomes mimicking the lipid composition of the Gram-negative inner bacterial membrane and an average composition of eukaryotic membrane were prepared following the protocol reported by Bonucci *et al*. [14].

Antimicrobial assay

E. coli MG1655 was used as the representative Gram-negative bacteria. *E. coli* was cultured in Tryptic Soy Broth (TSB) at 37 °C under shaking at 160 rpm. *S. agalactiae* (GBS) COH1 was used as representative capsulated Gram-positive bacteria. GBS was cultured in TSB at 37 °C without shaking. Bacteria with compromised membranes were detected by monitoring the fluorescence of the DNA binding dye SYTOX Green (INVITROGEN, Molecular Probes). GBS and *E. coli* grown at 37 °C in TSB media up to an optical density (OD) of about 0.6-0.8 (mid-logarithmic phase) were washed twice and re-suspended in TSB 10%. Peptides at the indicated concentrations were incubated with bacteria (1 x 10^7 CFU/mL) and SYTOX Green (5μg/mL) in 10% TSB at 37 °C in a black flat bottom 96-well plate. Negative control consisted of bacteria incubated in the same conditions as above described in the absence of peptides. Permeabilization of the bacterial cytoplasmic membrane allows the SYTOX Green dye to enter inside the cells and to intercalate with the DNA. When excited at 490 nm, the dye bound to DNA resulted in an increase in the emitted fluorescence at 535 nm, measured every 5 minutes in a microtiter plate reader (Tecan M200).

Cell culture

A549 cells were purchased from the American Type Culture Collection (Rockville MD) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum, glutamine and antibiotics as recommended by the vendor in a humidified atmosphere of 5% CO_2, 95% air at 37 °C. A549 cells were trypsinized and transferred to 96-well plates 24 h before treatment with LL-37.

Cell viability test

Cytotoxic effect of LL-37 and its variants was assessed by measuring cellular redox activity with 3-[4,5-dimethylthiazol -2-yl]-2,5-diphenyltetrazolium bromide (MTT) [17]. LL-37 was dissolved in sterilized 100 mM sodium phosphate buffer (pH 7.4). A549 cells were plated at a density of 10^4cells/100 μl per well in 96-well tissue culture plates. After 24-h incubation with phosphate-buffered saline (PBS, pH 7.4), peptide samples were added to the wells at different concentrations for 18 and 24 h. After treatment, the culture medium was removed and 10 μl of MTT (0.5 mg/ml) dissolved in the culture medium was added and incubated for 4 h at 37 °C. Plates were gently shaken for 10 min and the absorbance due to MTT reduction, representing cytotoxicity [18], was measured in a microtiter plate reader (Tecan M200).

RESULTS

Biophysical features and secondary structure of LL-37

To identify the cumulative effects exerted by multiple substitutions of Arg with Lys on biological activities we used LL-37 analogs already characterized [13]. As shown in Fig.1 each peptide keeps only one of the 5 Arg residues of LL-37, while the other 4 Arg residues are replaced by Lys. These are conservative mutations; therefore all these LL-37 analogs share the same

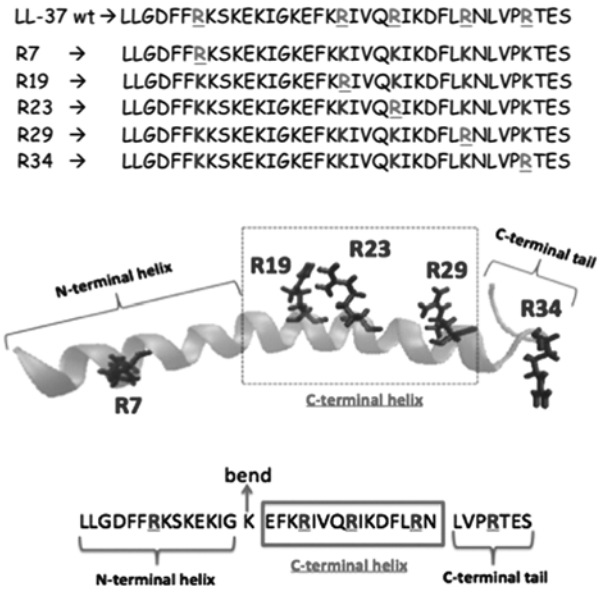

Figure 1. Amino acid sequences and secondary structure of LL-37. The amino-acid sequences of native LL-37 and variants and the mutated amino-acids are represented in the upper panel. In the lower panel the secondary structure of LL-37 is represented, with the key Arg residues shown in bold-face. The structure was obtained using Visual Molecular Dynamics and Discovery Studio tools (LL-37 2K60 pdb code).

length (37 amino-acid residues), net charge (+6) and hydrophobicity ratio (35) with the wild-type LL-37 (Fig. 1). In the CD spectra of R28 a negative absorbance band around 200 nm and a typical CD line-shape of unordered conformation was observed (Fig. 2). After addition of 50% (v/v) trifluoroethanol (TFE), the two additional peaks observed at ~208 and ~220 nm, respectively, indicated a change into a defined alpha-helix structure of the peptide. These data, also observed with the other mutants (data not shown), indicate that replacing Arg residues with Lys does not influence the folding of LL-37 in solution since similar results were obtained with wild-type LL-37 [14].

To understand the effect of multiple Lys substitutions on the interaction of LL-37 with negatively charged lipid bilayers, CD spectra of each mutant in the presence of model bacterial membranes at different peptide:lipid (pep:lip) molar ratio were recorded (Fig. 3). At low pep:lip molar ratio (1:250), CD spectra presented a line-shape corresponding to a mix of different secondary structures for all the peptides, except for R23 mutant, which presented a more defined alpha-helix arrangement (Fig. 3). Indeed, the simulation analysis for all the other mutants revealed that each peptide coexists in solution with unordered (~90%), alpha-helix (~6%) and beta-sheet (~4%) conformations. These results differ from those obtained with wild-type LL-37 indicating that the replacement of specific Arg residues affects the folding of LL-37 mutants into a defined structure in the presence of model bacterial membrane at 1:250 pep:lip molar ratio.

For higher pep:lip molar ratio ratios (1:50 and 1:20), CD spectra showed two negative absorbance peaks at ~208 nm and ~220 nm for each variant tested, suggesting a peptide folding into an alpha-helix structure on the lipid membrane surface. This behaviour observed for all mutants was similar to that previously found for wild-type LL-37 peptide [14]. However, Dichroweb analysis revealed that only ~75% of mutants in solution display a helix conformation as compared to ~92% of LL-37 in the same conditions. The substitution of Arg residues could partially influence the peptide to adopt a defined conformation on the membrane outer layer. At 1:5 pep:lip molar ratio, R7, R19, R23 and R29

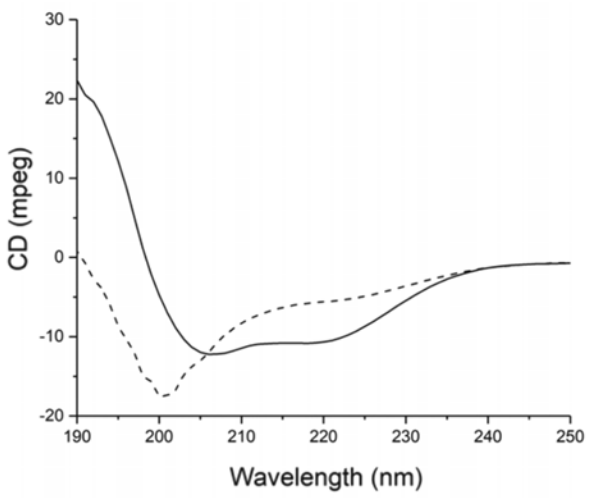

Figure 2. Circular dichroism of the R29 mutant. The circular dichroism (CD) spectra was recorded in MOPS buffer at pH 6.8 (dotted line) and in the presence of 50% (v/v) TFE (solid line). The other peptide variants tested in this work gave similar spectra (data not shown). Each spectrum was recorded at room temperature ten times in order to reduce signal-to-noise ratio.

mutants still show CD absorbance bands at ~208 nm and ~220 nm and a line-shape similar to those obtained for 1:50 and 1:20 molar ratios. In fact, the percentage of alpha-helix contribution was estimated to be in the 70-80% range for LL-37 variants. On the other hand, the R34 peptide showed different CD spectra at higher pep:lip molar ratio compared to the results obtained for the other mutants tested (Fig. 3). The typical peaks of helix structure are still evident but a net decrease in molar ellipticity was also recorded. Since only this variant showed a similar spectrum to wild-type LL-37, the presence of Arg residue at position 34 strongly affects the capability of this antimicrobial peptide to form aggregates on model bacterial membrane over the activity threshold ratio (1:50 pep:lip for native LL-37).

LL-37 variants have improved membrane permeation activity against *E. coli* and *S. agalactiae*

To investigate the kinetics of bacterial killing of LL-37 mutants we used a fluorescence-based assay. The time-dependent membrane permeation curves of peptides (at 12.5 μM concentration) against *E. coli* MG1655, as representative of the

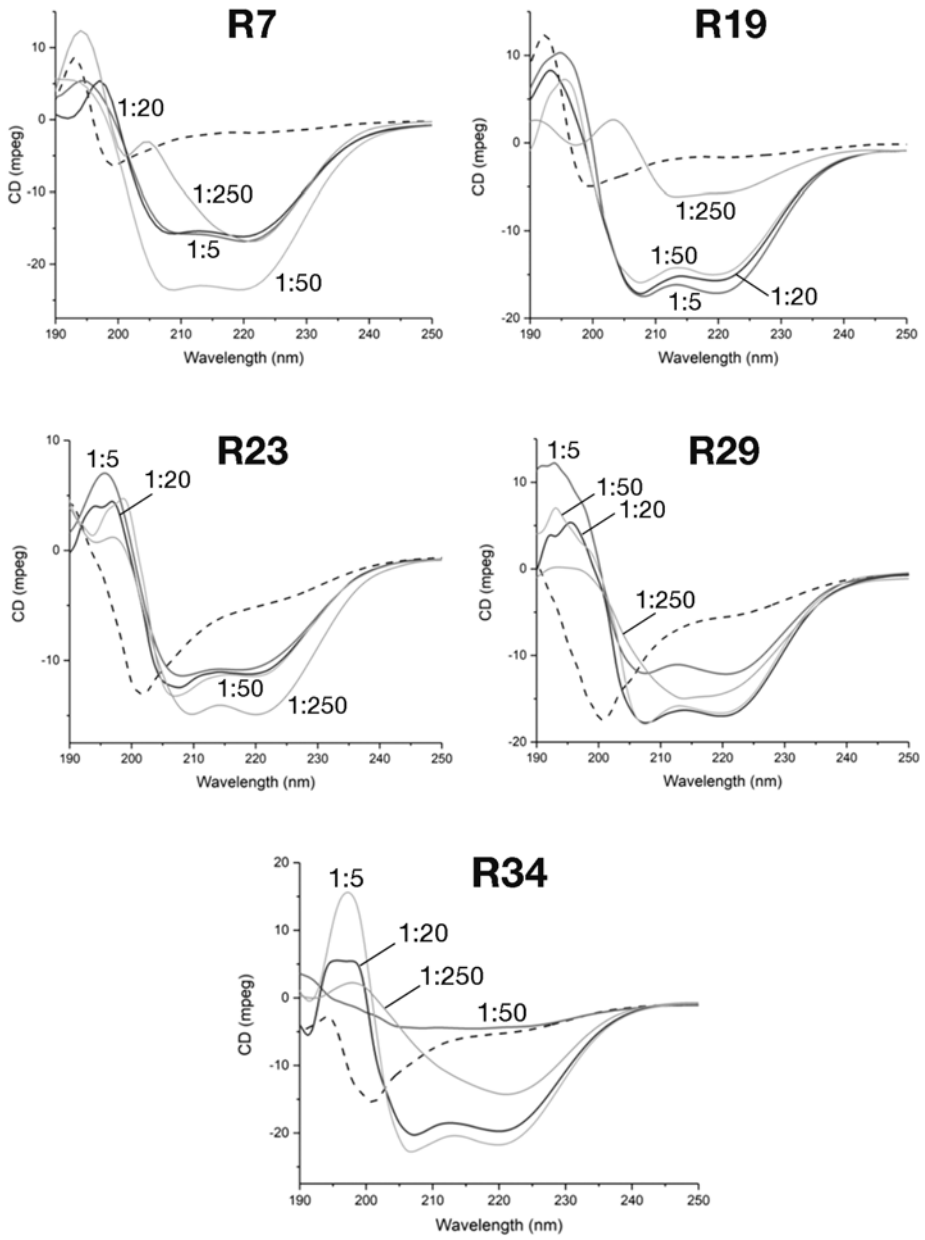

Figure 3. Circular dichroism of LL-37 mutants in the presence of model membranes. CD spectra of LL-37 variants were recorded in MOPS buffer at pH 6.8 (dotted line) and in the presence of POPE:POPG:CL LUVs at 1:250, 1:50, 1:20 and 1:5 peptide:lipid molar ratio, respectively. Each spectra was obtained at room temperature and measurements were repeated ten times to maximize signal to noise ratio.

Gram-negative bacteria, are shown in Fig. 4 (*upper panel*). While R19, R23 and R34 reached 100% killing activity earlier than wild-type LL-37, R7 reached it only after 1 hr of incubation while R29 did not show any bactericidal activity. The differences in the bactericidal efficiency of the different peptides are more evident after 30 min incubation, as shown in Fig. 4 (*lower panel*). At this time point R19, R23 and R34 showed a 2-fold increase in bactericidal activity as compared to wild-type peptide, R7 a reduced activity, while in R29 this effect was almost absent. *S. agalactiae* COH1 was used as a representative of capsulated Gram-positive bacteria.

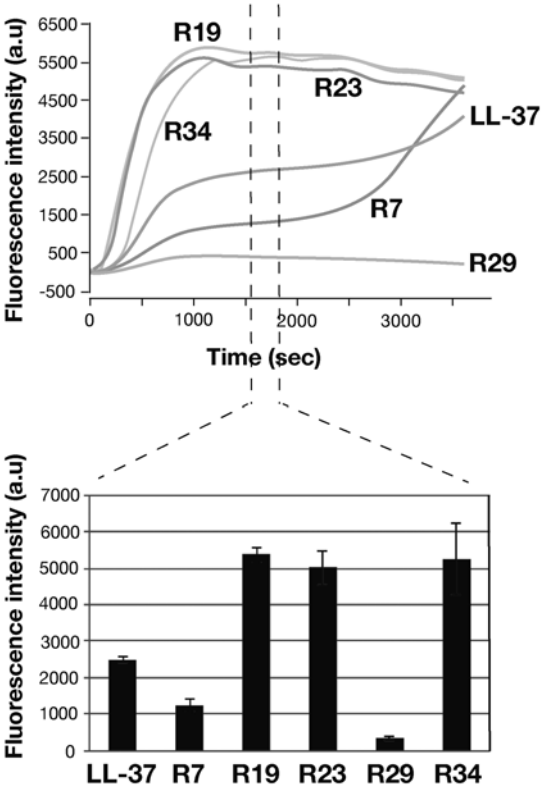

Figure 4. Antimicrobial activity of wild-type and mutated LL-37 against *E. coli*. Bacterial membrane damage was detected by monitoring the fluorescence of the DNA binding dye SYTOX Green, as described in the Material and Methods section. *E. coli* MG1655 were grown at 37 °C in TSB media and then incubated with 12.5 μM LL-37 wild-type or mutated up to 1 h (upper panel). Bacteria incubated in the absence of peptides represented the negative control (not shown). Bacterial membrane damage after 30 min. incubation with peptides represented as the mean (SD) of fluorescent intensity values of three independent experiments is shown in the lower panel.

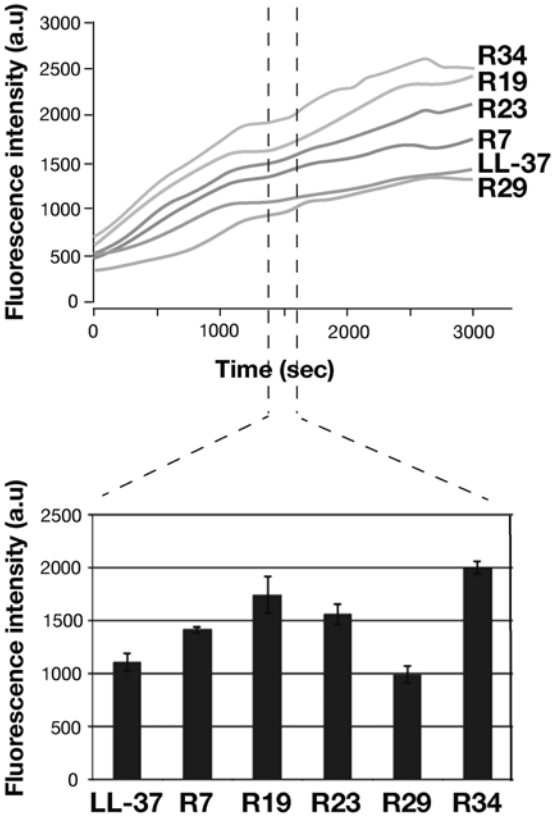

Figure 5. Antimicrobial activity of LL-37 and variants against *S. agalactiae*. Antimicrobial activity was detected by monitoring the fluorescence of the DNA binding dye SYTOX Green as described in the 'Materials and Methods' section. Results of *Streptococcus agalactiae* COH1 incubated with 100 μM LL-37 and variants are shown (upper panel). Membrane permeation of *Streptococcus agalactiae* COH1 incubated with 100 μM LL-37 and variants for 30 min is shown in the lower panel. The values are expressed as the mean of three independent experiments.

The overall bactericidal activity was lower as compared to the *E.coli*, probably due to the presence of the capsule, and required an 8-fold higher concentration than that used for *E.coli* (100 μM, Fig. 5 *upper panel*). In analogy with what was previously observed, R19, R23 and R34 maintained the same effect against *S. agalactiae* (Fig. 5). Differently from what was observed before, R7 is slightly more bactericidal with R29 keeping the same intensity of LL-37. This is clearly evidenced in Fig. 5 (*lower panel*) after 30 min of incubation.

LL-37 variants with enhanced antimicrobial activity have lower cytotoxic activity than wild-type LL-37

In our previous paper we demonstrated that LL-37 assumes an oligomeric conformation in the presence of neutral membranes [14]. This is in agreement with the lack of cell selectivity of LL-37 compared to other α-helical amphipatic antimicrobial peptides. Therefore, at high concentration LL-37 can also be cytotoxic versus eukaryotic cells. With the hope of dissociating bactericidal and cytotoxic effects of LL-37 we

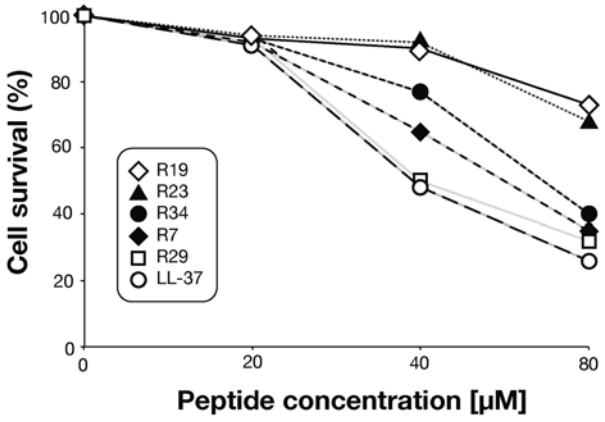

Figure 6. Dose response killing effects of LL-37 and variants on the viability of A549. A549 cells were cultured at a concentration of 10^4/cells per well. Viability was measured by incubating LL-37 (○) and variants R7 (◆), R19 (◇), R23 (▲), R29 (□), and R34 (●) at 20, 40 and 80 μM for 18 h. The cytotoxicity was measured with the MTT assay as described in the Materials and Methods section. Results are expressed as the percentage of cell viability of treated group with respect to untreated control group. Data shown are representative of one out of three independent experiments with similar results.

tested the cell viability of LL-37 variants and wild-type on a cell line. Since epithelial cells are likely affected by LL-37 released in the milieu, we used the A549 human epithelial cell line [19]. A549 cells were incubated with increasing concentrations of peptides for 18 and 24 h. As shown in Fig. 6 after 18 h of incubation, cell viability declined in a concentration-dependent manner for LL-37 and R29. Peptides R19 and R23 caused significantly less cell lysis than LL-37 and R29 at each concentration used. Peptides R7 and R34 were slightly less cytotoxic than LL-37 at 40 μM but comparable to LL-37 at 80 μM (Fig. 6). A similar decline in cell viability was also observed after 24 h of incubation (data not shown).

DISCUSSION

The electrostatic and hydrophobic interactions between amino acids and phospholipid head groups and alkyl chains are considered as the principal driving force of antimicrobial effects of AMPs. In the last few years several studies analyzed different fragments of LL-37 and some variants to assess the importance of amino acid position on the mechanism of action against lipid membranes [20]. It has emerged that the central core of cathelicidin peptide, which contains Arg at position 19, 23 and 29, is the principal region responsible for the interaction with lipid bilayer. In particular Arg 23, in combination with Lys 25, turned out to be fundamental for the interaction with PG lipid and penetration into the outer layer [21]. On the other hand, Arg 19 and Arg 29 displayed a secondary effect of lipid clustering and bacterial aggregation; these effects are still important for LL-37 activity.

In order to characterize the importance of these residues in LL-37 biological and biophysical properties, we recorded the CD spectra of several mutants of LL-37 peptide that contain a multiple substitution of Arg with Lys, both in buffer media and in the presence of model bacterial membranes at various pep:lip molar ratio. Our results indicate that each Lys mutant displays the capability to fold into alpha-helix conformation in the presence of a model bacterial membrane at 1:50 and 1:20 pep:lip molar ratios, while at low ratio (1:250 pep:lip) it is still prevalently unordered in solution. This behaviour was similar to that previously found for native LL-37 [14]. However, the propensity of all variants to assume a defined conformation on the membrane surface was slightly lower than wild-type LL-37. This effect is related to the Lys substitution that could alter the cooperative mechanism of several amino acids responsible for anchoring lipids and the consecutive folding and aggregation process.

For the highest ratio (1:5 pep:lip) R7, R19, R23 and R29 still possess a helix conformation in the presence of large unilamellar vesicles (LUVs), contrary to our finding on LL-37 where the formation of aggregates on lipid bilayer was observed. Nevertheless, R34 was the only mutant, which reveals a mechanism of aggregation in this condition. This outcome is in agreement with various studies that reported position 34, and in general the C-terminal tails of LL-37, as the region mainly involved in peptide oligomerization.

Surprisingly, measurements of membrane permeation of mutants against *E.coli* evidenced a higher microbicidal activity for R19, R23 and R34 mutants compared to native LL-37. Comparing

biophysical and microbiological findings, we can state that Arg on position 19, 23 and 34 are crucial for antibacterial activity. In addition, the formation of aggregates on membrane surface at high ratio (not revealed for R19 and R23 by CD experiments) probably is not a pivotal feature for LL-37 killing activity of Gram-negative bacteria. Such a difference may correlate to membrane composition, since Gram-negative bacteria possess an outer membrane, which is absent in Gram-positive bacteria. Contrary, R7 and R29 demonstrated a reduced and almost absent *E.coli* disrupting activity, respectively, compared to wild-type LL-37 peptide. For R19 and R29 located on the hydrophilic face of the amphipathic structure, we observed a different activity. While the Arg-Lys swap in position 29, reduced the biocidal activity, the Arg-Lys swap in position 19 enhanced this effect. Lys to Arg substitutions proved to be favourable for bacterial killing against *E.coli* [21]. In our hands, except for R29, there is an increased biocidal activity. A possible reason for these apparent conflicting results is that a combination of Arg and Lys in key residues, which are located at the interface of helical structure such as R23 (Fig. 1), could be beneficial for cationic peptides. An effective antimicrobial agent should not only exert antimicrobial activity but also reduce its toxicity towards host cells. While R34, which is more active towards *E.coli* shows comparable toxicity towards A549 cells at 80 μM, herein R19 and R23 analogs showed low toxicity at 40 and 80 μM. The difference with the high toxicity of parent peptide LL-37 is evident. Cancer cells are usually more easily targeted by cationic peptides due to the membrane composition. However the low toxicity of the two LL-37 analogs tested might suggest that multiple mutations with unconventional amino acids could be used to improve biological properties of cationic peptides.

CONCLUSION

In conclusion the Arg to Lys mutational effects is strictly dependent on the residue position and nature of peptide since in some cases these mutations produce little change in some properties. The findings shown herein could be relevant in better understanding the relationship between amino acids and their sequence positions and could be of help when programming the synthesis of new antimicrobials.

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CONFLICT OF INTEREST STATEMENT

The authors report no financial conflict of interest.

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