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ORIGINAL ARTICLE

D-Amino acids incorporation in the frog skin-derived peptide esculentin-1a(1-21)NH_2 is beneficial for its multiple functions

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Abstract Naturally occurring antimicrobial peptides (AMPs) represent promising future antibiotics. We have previously isolated esculentin-1a(1-21)NH_2, a short peptide derived from the frog skin AMP esculentin-1a, with a potent anti-*Pseudomonal* activity. Here, we investigated additional functions of the peptide and properties responsible for these activities. For that purpose, we synthesized the peptide, as well as its structurally altered analog containing two D-amino acids. The peptides were then biophysically and biologically investigated for their cytotoxicity and immunomodulating activities. The data revealed that compared to the wild-type, the diastereomer: (1) is significantly less toxic towards mammalian cells, in agreement with its lower α-helical structure, as determined by circular dichroism spectroscopy; (2) is more effective against the biofilm form of *Pseudomonas aeruginosa* (responsible for lung infections in cystic fibrosis sufferers), while maintaining a high activity against the free-living form of this important pathogen; (3) is more stable in serum; (4) has a higher activity in promoting migration of lung epithelial cells, and presumably in healing damaged lung tissue, and (5) disaggregates and detoxifies the bacterial lipopolysaccharide (LPS), albeit less than the wild-type. Light scattering studies revealed a correlation between anti-LPS activity and the ability to disaggregate the LPS. Besides shedding light on the multifunction properties of esculentin-1a(1-21)NH_2, the D-amino acid containing isomer may serve as an attractive template for the development of new anti-*Pseudomonal* compounds with additional beneficial properties. Furthermore, together with other studies, incorporation of D-amino acids may serve as a general approach to optimize the future design of new AMPs.

Keywords Diastereomer · Wound healing · Anti-biofilm activity · Antimicrobial peptide · Esculentin-1

Abbreviations

AMP	Antimicrobial peptide
CD	Circular dichroism
CFU	Colony-forming units
DMEMg	Dulbecco's modified Eagle's medium supplemented with glutamine
FBS	Heat-inactivated fetal bovine serum
LB	Luria–Bertani broth
LPC	Lysophosphatidylcholine
LPS	Lipopolysaccharide
MTT	3(4,5-Dimethylthiazol-2yl)2,5-diphenyltetrazolium bromide
PBS	Phosphate buffered saline

Introduction

During the past three decades, the widespread use of conventional antibiotics has led to a drastic reduction in their

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This manuscript is dedicated to Professor Emeritus Donatella Barra who passed away on September 28th, 2014.

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therapeutic efficacy with over 70 % of hospital bacterial infections being resistant to them (Chen et al. 2009; Grundmann et al. 2011; Kaye 2012). This has resulted in more difficult and costly treatments, longer hospitalization, higher risk of complications and increase in death. Furthermore, the number of new antibiotics approved by the FDA has steadily declined every year since 1980 and most therapeutics in clinical development are derivatives of existing medicines (Lohner and Staudegger 2001). Hence, the discovery of new classes of antimicrobial agents with a new mode of action is a major challenge. Cationic antimicrobial peptides (AMPs) are ancient elements of the innate immune defense of all living organisms (Boman 1995; Mangoni 2011; Yeung et al. 2011; Bevins 2013; Kai-Larsen et al. 2014; Mansour et al. 2014) with common properties, e.g., a net positive charge at neutral pH and an amphipathic character in a hydrophobic environment (Epand and Vogel 1999; Shai 2002). They hold promise for the generation of new drugs (Hancock and Sahl 2006; Dempsey et al. 2010; Alba et al. 2012; Guralp et al. 2013; Haney and Hancock 2013) and are considered as future compounds against both acute and chronic microbial infections (Brown and Hancock 2006; Cruz et al. 2014). Of particular interest are those caused by the multidrug-resistant biofilm-forming gram-negative bacterium *Pseudomonas aeruginosa* in the lungs of cystic fibrosis (CF) sufferers (Drenkard and Ausubel 2002; Bjarnsholt et al. 2009; Millar et al. 2009; Macia et al. 2014). In these patients, the lack of Cl^- secretion promotes airways drying and mucus plugging, predisposing the lungs to microbial colonization, deterioration of lung tissue and impairment of respiratory functions (Moreau-Marquis et al. 2008). Since the mechanism of killing by the majority of AMPs includes an irreversible membrane injury, it makes it difficult for the bacteria to develop resistance against them (Shai 2002; Mangoni 2006; Mangoni et al. 2007), although some AMPs have been reported to induce limited resistance (Peschel et al. 1999). Noteworthy, antibiotic treatment of gram-negative bacteria is accompanied with the release of bacterial cell wall components, such as the lipopolysaccharide (LPS), also named endotoxin. LPS activates immune cells (Rietschel et al. 1994), leading to the secretion of pro-inflammatory cytokines e.g., tumor necrosis factor-alpha (TNF-α) whose high levels can damage lung functionality and cause septic shock syndrome, in the most serious cases (Poltorak et al. 1998; Cohen 2002). Regarding this, several AMPs have already shown additional properties, including the capability to neutralize the toxic effect of LPS (Hancock et al. 2012; Pulido et al. 2012). This makes them important modulators of both adaptive and innate immunity, and distinguishes them from traditional antibiotics (Hancock and Sahl 2006; Fjell et al. 2012; Hancock et al. 2012; Semple and Dorin 2012).

Recent studies demonstrated that esculentin-1a(1-21) NH_2 [Esc(1-21)] derived from the N-terminal region of the 46 residues frog skin AMP esculentin-1a (Gamberi et al. 2007; Islas-Rodriguez et al. 2009) is endowed with antimicrobial activity at high ionic concentrations, which is a crucial aspect due to the high-salt environment existing at the apical side of CF epithelial cells. In addition, in vivo experiments revealed that Esc(1-21) prolongs survival of murine models of *P. aeruginosa*-induced sepsis or lung infection, upon intravenous or intra-tracheal administration, respectively (Luca et al. 2013). Despite this and other studies showing an in vivo antimicrobial activity of AMPs (Giacometti et al. 2006; Pini et al. 2010; Uccelletti et al. 2010; Xiong et al. 2011; Luca et al. 2014; Jung Kim et al. 2014; Kolar et al. 2015), inherent limitations for the development of these molecules as new anti-infective therapeutics include: (1) cytotoxicity outside their natural environment, which can strongly dampen the in vivo antimicrobial efficacy of AMPs, and (2) problems with stability, bioavailability and delivery systems. With reference to the latter, studies on the effect of the incorporation of D-amino acids into AMPs were mainly focused on the antimicrobial activity of the peptides, their reduced cytotoxicity and their increased stability to circulating enzymes (Hamamoto et al. 2002; Papo et al. 2002). However, before AMPs can be clinically used, evaluation of other beneficial properties (e.g., anti-inflammatory, wound healing) should be also investigated. Here, we report on the synthesis and properties of a diastereomer of Esc(1-21), named Esc-1a(1-21)-1cNH_2 [Esc(1-21)-1c], containing two D-amino acids, and compared its multiple functions with those of the wild-type Esc(1-21). The results are discussed in line with the advanced properties of the diastereomer as a potential non-toxic wound healing promoter in addition to its antimicrobial activity.

Materials and methods

Materials

Trypsin–EDTA was purchased from Invitrogen (Life-Technologies Europe, Monza, Italy); 3(4,5-dimethylthiazol-2yl)2,5-diphenyltetrazolium bromide (MTT), Triton X-100, AG1478, mitomycin C, Hoechst 33258, LPS from *P. aeruginosa* serotype 10, lysophosphatidylcholine (LPC, (1-0-palmitoyl-sn-glycero-3-phosphocholine) were all from Sigma–Aldrich (St. Luis, MO). Dulbecco's modified Eagle's medium (DMEM); non-essential amino acids (NEAA); sodium pyruvate (100 mM); heat-inactivated fetal bovine serum (FBS); glutamine; penicillin/streptomycin were from Euroclone (Milan, Italy). All other chemicals were reagent grade.

Peptides synthesis

Synthetic Esc(1-21) and the diastereomer Esc(1-21)-1c were purchased from Selleck Chemicals (Houston, TX, USA). Briefly, the peptides were assembled by step-wise solid-phase synthesis by a standard F-moc strategy and purified by RP-HPLC on a semipreparative C18-bonded silica column (Kromasyl, 5 μm, 100 Å, 25 cm × 4.6 mm) using a gradient of acetonitrile in 0.1 % aqueous trifluoroacetic acid at a flow rate of 1.0 ml/min, according to what reported in (Luca et al. 2013). Analytical RP-HPLC indicated a purity >98 %. The molecular mass was verified by using MALDI-TOF Voyager DE (Applied Biosystems) as previously described (Islas-Rodriguez et al. 2009).

Peptide stability

A total of 125 μl of a 0.92 mM solution of peptide was incubated at 37 °C with 20 and 60 μl human serum. Samples were collected from healthy volunteers after 5 and 24 h of incubation, precipitated with 200 μl methanol, and centrifuged for 2 min at 10,000×*g*. The crude solution was then analyzed by HPLC and mass spectrometry. HPLC was performed with a Vydac C18 column, and the crude solution was diluted 5 times with 0.1 % trifluoroacetic acid before injection, and monitored at 280 nm.

Microorganisms

The strains of *P. aeruginosa* used for the antimicrobial assays were: the standard non mucoid ATCC 27853 (Li et al. 2001), and the following ones from collection of the CF clinic Medizinische Hochschule of Hannover, Germany: the mucoid AA11 and the non-mucoid TR1 and KK1 (Bragonzi et al. 2009).

Cell culture

The murine Raw 264.7 macrophage cell line and the human type II alveolar epithelial cell line A549 (from the American Type Culture Collection, Manassas, Va) were employed (Akram et al. 2013). Cells were cultured in DMEM supplemented with 10 % FBS, glutamine (2 mM), and antibiotics (0.1 mg/ml of penicillin and streptomycin) at 37 °C and 5 % CO_2 in 25-cm^2 flasks. In the case of macrophages, NEAA and sodium pyruvate were also added to the culture medium.

Antimicrobial activity

Bacteria were grown in Luria-Bertani (LB) broth at 37 °C till a mid-log phase which was aseptically monitored by absorbance at 590 nm (A_{590nm} = 0.8) with an UV-1700 Pharma Spec spectrophotometer (Shimadzu, Tokyo, Japan). Afterwards, bacterial cells were centrifuged at 1400×*g* for 10 min and resuspended in phosphate buffered saline (PBS).

About 1 × 10^5 colony-forming units (CFU) in 100 μl PBS were incubated at 37 °C with Esc(1-21) or Esc(1-21)-1c in serial twofold dilutions. Aliquots of 10 μl were withdrawn at different time intervals, diluted in LB and spread onto LB-agar plates. After overnight incubation at 37 °C, the number of CFU was counted. Controls were run without peptide and in the presence of peptide solvent (Luca et al. 2013).

Bactericidal activity was defined as the peptide concentration necessary to cause a reduction in the number of viable bacteria of ≥3 $\log_{10}$ CFU/ml.

Antibiofilm activity

Biofilm formation was performed by adapting the procedure described in (Ceri et al. 2001; Falciani et al. 2012), using the Calgary Biofilm Device (Innovotech, Innovotech Inc. Edmonton, Canada). Briefly, 96-well plates, each well containing 150 μl of the bacterial inoculum (1 × 10^7 CFU/ml) in LB medium were sealed with 96 peg-lids on which biofilm cells can build up. Afterwards, plates were placed in an humidified orbital incubator at 35 °C for 20 h under agitation at 125 rpm. Once biofilms were allowed to form, the pegs were rinsed twice with PBS to remove planktonic cells. Each peg-lid was then transferred to a "challenge 96-well microtiter plate", each well containing 200 μl of a twofold serial dilution of peptide in PBS. The "challenge plate" was incubated at 37 °C for 2 h. Peptide activity was evaluated by determining the amount of viable biofilm cells by measuring the reduction of MTT to its insoluble formazan. Briefly, after peptide treatment, the pegs-lid was washed with PBS and used to close another 96-well microtiter plate, each well containing 200 μl of Hank's buffer (136 mM NaCl; 4.2 mM Na_2HPO_4; 4.4 mM KH_2PO_4; 5.4 mM KCl; 4.1 mM $NaHCO_3$, pH 7.2, supplemented with 20 mM D-glucose) containing 1 mg/ml MTT. The plate was incubated at 37 °C for 4 h. Afterwards, 50 μl of 25 % sodium dodecyl sulfate were added to each well to dissolve formazan crystals. Bacterial viability was determined by absorbance measurements at 595 nm and calculated with respect to control cells (bacteria not treated with the peptide) (Mangoni et al. 2005). The percentage of viable cells was calculated according to the equation:

$$\frac{\text{Absorbance}_{\text{sample}} - \text{Absorbance}_{\text{blank}}}{\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{blank}}} \times 100$$

where the blank is given by samples without cells and not treated with the peptide.

Peptides' effect on cell viability

The effect of both peptides on the viability of mammalian macrophages and lung epithelial cells was determined by the MTT assay according to (Di Grazia et al. 2014). Lung epithelial cells in DMEM supplemented with 2 mM glutamine (DMEMg) and 2 % FBS or macrophages in DMEMg containing NEAA, sodium pyruvate and 2 % FBS were plated in triplicate wells of a microtiter plate, at 4×10^4 cells/well. After overnight incubation at 37 °C in a 5 % CO_2 atmosphere, the medium was replaced with 100 µl fresh serum-free medium supplemented with each peptide at different concentrations. The plate was incubated for 24 h at 37 °C in a 5 % CO_2 atmosphere. Afterwards, the medium was removed and replaced with Hank's buffer containing 0.5 mg/ml MTT. After 4 h incubation at 37 °C, the formazan crystals were dissolved by adding 100 µl of acidified isopropanol according to (Grieco et al. 2013), and viability was determined by absorbance measurements at 570 nm using a microplate reader (Infinite M200; Tecan, Salzburg, Austria). Cell viability was calculated with respect to the control (cells not treated with peptide). The percentage of viable cells was calculated as described above. LD_{50}, is the lethal peptide dose causing 50 % reduction in the number of viable cells.

Cell migration assay

The ability of both peptides to stimulate migration of lung epithelial cells was studied as follows: A549 cells (4×10^4) suspended in DMEMg and supplemented with 10 % FBS were seeded on each side of an Ibidi culture insert for wound healing assay (Ibidi, Munich, Germany). Inserts were placed into 35-mm dish plates and incubated overnight at 37 °C and 5 % CO_2 to allow cells grow to confluence. Afterwards, inserts were removed to create a cell-free area (pseudo-"wound") of approximately 500 µm; 1 ml DMEMg supplemented with 2 % FBS and containing or not the peptide at different concentrations was added. Cells were allowed to migrate in an appropriate incubator. At 0, 15, 20 and 24 h, fields of the injured area were visualized microscopically under an inverted microscope (Olympus CKX41) at 4× magnification and photographed with a Color View II digital camera. The percentage of cell-covered area at each time was determined by WIMASIS Image Analysis program. Pseudo-"wound" closure assays were also conducted after cells pretreatment with the cell proliferation inhibitor mitomycin C (5 µM) for 90 min (Chieng-Yane et al. 2011; Wang et al. 2012). Furthermore, the involvement of epidermal growth factor receptor (EGFR) in peptide-induced cell migration was analyzed by pretreating cells for 15 min with 0.2 µM AG1478 inhibitor (Tokumaru et al. 2005; Hoq et al. 2011).

Fluorescence studies

Lung epithelial cells (1.5×10^5) were seeded on coverslips (properly put into 35 mm dishes) in DMEMg supplemented with 10 % FBS, at 37 °C and 5 % CO_2. After overnight incubation, cells were washed with PBS and pretreated or not with 0.2 µM AG1478 before adding 10 µM Esc(1-21) or 4 µM Esc(1-21)-1c in DMEMg supplemented with 2 % FBS. For comparison, cells incubated with 0.2 µM AG1478 without any peptide treatment were also included. Cells neither treated with the peptide nor with the inhibitor served as control. After 24 h incubation at 37 °C and 5 % CO_2, cells were washed with PBS and fixed with 3.7 % formaldehyde for 10 min at 4 °C. Afterwards, they were washed with PBS, permeabilized with 0.1 % Triton X-100 in PBS for 10 min at room temperature, washed again and stained with phalloidin-fluorescein isothiocyanate (40 µM in PBS) for 30 min at room temperature to visualize the cytoskeleton. The nuclei were stained by adding 50 µl of Hoechst 33258 (2 µg/ml) for 10 min at room temperature. The coverslips were mounted on slides using buffered glycerol, observed under the fluorescent microscope KOZO OPTICS XJF800 at 20× magnification and photographed with a Color View II digital camera.

Evaluation of Esc(1-21) isomers on the TNF-α release from Raw 264.7 macrophages

Macrophages were cultured overnight in 96-well plates (1×10^5 cells/well) in DMEMg supplemented with sodium pyruvate, NEAA and 10 % FBS. The medium was then removed and replaced with fresh medium containing 10 ng/ml LPS in the presence of 1, 5, 10 and 20 µM peptide. Samples were incubated at 37 °C for 4 h. The medium was then collected and TNF-α concentration was evaluated using a mouse TNF-α enzyme-linked immunosorbent assay kit according to the manufacturer's protocol (eBioscience, Affymetrix, San Diego, CA, USA). Cells that were stimulated with LPS alone and untreated cells served as controls. All experiments were done in triplicates.

Circular dichroism (CD) spectroscopy

CD measurements were performed on an Aviv 202 spectropolarimeter (Applied Photophysics spectropolarimeter, United Kingdom). The spectra were scanned using a thermostatic quartz cuvette with a path length of 1 mm. All measurements were done at 25 °C. The average time recording of each spectrum was 20 s in 1 nm steps in the wavelength range of 190–260 nm. The peptides were scanned at a concentration of 50 µM in 5 mM Hepes, 50 µM of purified *P. aeruginosa* LPS and 10 mg/ml LPC. Average MW of LPS used for calculations is 4 kD.

Table 1 Peptide amount after 5 h and 24 h incubation with human fresh serum at 37 °C

Peptide designation	Peptide sequence[a]	Peptide amount (%)[b]			
		5 h		24 h	
		10 % Serum	30 % Serum	10 % Serum	30 % Serum
Esc-1a(1-21)NH_2	Gly-Ile-Phe-Ser-Lys-Leu-Ala-Gly-Lys-Lys-Ile-Lys-Asn-Leu-Leu-Ile-Ser-Gly-Leu-Lys-Gly-NH_2	44.4	20.95	22.19	11.5
Esc-1a(1-21)-1cNH_2	Gly-Ile-Phe-Ser-Lys-Leu-Ala-Gly-Lys-Lys-Ile-Lys-Asn-***Leu***-Leu-Ile-***Ser***-Gly-Leu-Lys-Gly-NH_2	63.34	30.12	45.61	25.46

[a] D-Amino acids are in italics and bold

[b] Peptide amounts were determined by the peak areas of the RP-HPLC relative to those of the control peptide (dissolved in PBS) at 0 min (set as 100 %)

LPS micelles measurements by using dynamic light scattering (DLS)

DLS is a well-known technique used to measure the Brownian motion (diffusion) and the size distribution of particles in solution. Here, we used the DLS machine (802 DLS manufactured by Viscotek) to determine the size of LPS micelles in solution. To estimate the average size of the LPS particles, the measurements were done in wavelength of 830 nm, the detection was performed at 90°, temperature between 22 and 25 °C (the refractive index was measured, respectively) and analyzed by the Software of OMNISIZE (Viscotek). LPS measurement was performed before and after peptides addition. LPS:peptide molar ratio was 1:1 and the solvent was filtered water.

Statistical analyses

Data were collected from three independent experiments. Quantitative data are expressed as the mean ± SEM. Statistical analysis was performed using two-way analysis of variance (ANOVA), with PRISM software (GraphPad, San Diego, CA). Differences were considered to be statistically significant for $p < 0.05$. The levels of statistical significance are indicated in the legend to figures.

Results

Design of Esc(1-21)-1c

Previous studies using CD spectroscopy showed that Esc(1-21) adopts an α-helical structure in phosphatidylethanolamine/phosphatidylglycerol (7:3, v:v) vesicles mimicking the lipid composition of the cytoplasmic membrane of gram-negative bacteria (Epand et al. 2006, 2007). Similar results were found for the shorter analog, Esc-1b(1-18)NH_2 (Gly-Ile-Phe-Ser-Lys-Leu-Ala-Gly-Lys–Lys-Leu-Lys-Asn-Leu-Leu-Ile-Ser-Gly-NH_2) (Marcellini et al. 2009; Manzo et al. 2012), which is less active against gram-negative bacteria, e.g., *P. aeruginosa*, and differs from Esc(1-21) by a single amino acid at position 11, and lacks the 3 residues tail Leu-Lys-Gly-NH_2 at its carboxyl end.

In order to choose a rational position for incorporating D-amino acids in Esc(1-21), we took advantage of the available structural data on the shorter Esc-1b(1-18)NH_2. Solution-state NMR experiments in the presence of anisotropic membrane models, i.e., the negatively charged sodium dodecylsulfate/dodecylphosphocholine detergent micelles, revealed that Esc-1b(1-18)NH_2 consists of two helical segments separated by a kink at Gly^8 (Manzo et al. 2014). In addition, it was suggested that the cationic residues located at the N-terminal half of this peptide are important not only for peptide–bacteria binding but also for facilitating a deeper insertion of Esc-1b(1-18)NH_2 between the detergent headgroups. Furthermore, in the presence of zwitterionic micelles, which mimic the electrically-neutral membrane of mammalian cells only the C-terminal fragment was expected to fold in a helical conformation (Manzo et al. 2014). Therefore, with the aim of optimizing the biostability of Esc(1-21) without affecting its antimicrobial activity, we synthesized a diastereomer of Esc(1-21), named Esc(1-21)-1c, by replacing two L-amino acids in the C-terminal portion, i.e. $L\text{-}Leu^{14}$ and $L\text{-}Ser^{17}$, with the corresponding D-amino acid enantiomers (Table 1). These changes were also expected to decrease the toxicity of the peptide towards mammalian cells, by disturbing the expected C-terminal α-helix which is important for cytotoxicity (see "Discussion").

The diastereomer Esc(1-21)-1c is more stable than the all-L Esc(1-21) in human serum

The stability of both Esc(1-21) and its diastereomer in biological fluids were examined in the presence of 10 or 30 % fresh human serum after 5 and 24 h incubation at 37 °C. The data revealed that the isomer containing

Table 2 Anti-*Pseudomonal* activity of Esc(1-21) isomers

Bacterial strains	Bactericidal activity[a] (μM)	
	Esc(1-21)	Esc(1-21)-1c
P. aeruginosa ATCC 27853	1	4
P. aeruginosa AA11	1	4
P. aeruginosa KK1	1	4
P. aeruginosa TR1	1	4

[a] Bactericidal activity was defined as the lowest peptide concentration that is sufficient to reduce the number of planktonic viable bacteria by ≥ 3 $\log_{10}$ CFUs/mL in 30 min. The results are the average of four independent experiments

Table 3 Anti-biofilm activity of Esc(1-21) isomers

Bacterial strains	Anti-biofilm activity[a] (μM)	
	Esc(1-21)	Esc(1-21)-1c
P. aeruginosa ATCC 27853	12.5	12.5
P. aeruginosa AA11	25	12.5
P. aeruginosa KK1	12.5	12.5
P. aeruginosa TR1	25	12.5

[a] Antibiofilm activity was defined as the lowest peptide concentration that is sufficient to cause 95 % reduction in the amount of viable biofilm cells in 2 h. The results are the average of three independent experiments

D-amino acids, after 24 h, was less degraded compared to the wild-type: ~46 and 25 % of peptide in 10 and 30 % serum, respectively (Table 1). In comparison, the non-degraded amount of the wild-type Esc(1-21) after 24 h incubation was only ~22 and 11.5 %, in 10 and 30 % serum, respectively. Mass spectrometry analysis of the samples confirmed the presence of two main peaks, one at 2186 Da, corresponding to the calculated molecular mass of unmodified Esc(1-21)/Esc(1-21)-1c, and one at 495 Da corresponding to the secondary product (data not shown). Note that the majority of naturally occurring AMPs have very short (1-2 h) half-life, mainly because they are degraded by circulating proteases (Noto et al. 2008; Knappe et al. 2010; Nguyen et al. 2010; Falciani et al. 2012).

Esc(1-21)-1c is more active than Esc(1-21) against *P. aeruginosa* biofilms

The antibacterial activity of the two esculentin derivatives against the planktonic form of several strains of *P. aeruginosa* (either reference or clinical isolates from CF patients) was determined in phosphate buffered saline (Table 2). The data indicated that both isomers, at low concentrations, had potent microbicidal activity. The wild-type, at 1 μM, reduced ≥ 3 $\log_{10}$ the number of viable bacterial cells within 30 min, compared to the corresponding control (untreated cells). The diastereomer had the same efficacy at a concentration of 4 μM against all the examined bacteria (Table 2). Since *P. aeruginosa* biofilm growing is associated with the persistent chronic infection and colonization of lungs in CF patients, the two peptides were also tested for their ability to kill the sessile form of *P. aeruginosa* strains. As shown in Table 3, Esc(1-21)-1c displayed a higher anti-biofilm activity than the all-L peptide on both the clinical CF isolates AA11 and TR1, giving rise to 95 % reduction in the amount of viable biofilm cells at 12.5 μM compared to 25 μM found for Esc(1-21).

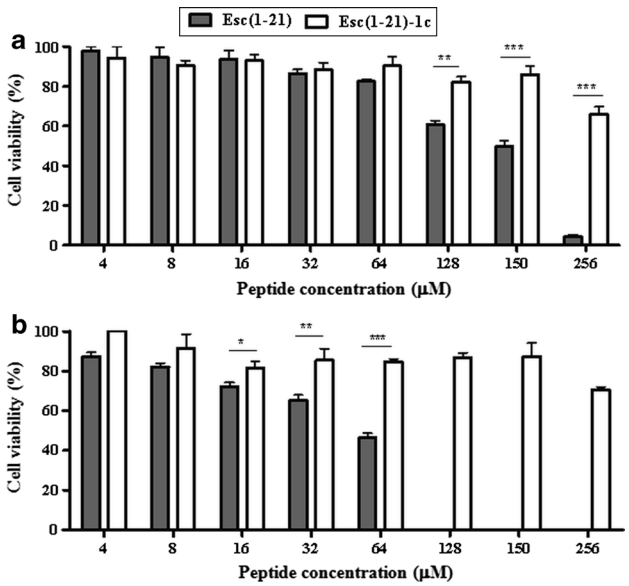

Fig. 1 Peptides' effect on the viability of A549 cells and Raw 264.7 macrophages. A549 cells (**a**) or macrophages (**b**) were plated in wells of a microtiter plate, at 4×10^4 cells/well in the corresponding culture medium (see "Materials and methods"). After overnight incubation at 37 °C in a 5 % CO_2 atmosphere, the medium was replaced with 100 μl fresh medium supplemented with the peptides at different concentrations. After 24 h of peptide treatment, cell viability was determined by the MTT reduction to insoluble formazan. Cell viability is expressed as percentage with respect to the control (cells not treated with the peptide). Data points represent the mean of triplicate samples ± SEM. The level of statistical significance between samples treated with Esc(1-21) and Esc(1-21)-1c are indicated as follows $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

The diastereomer is significantly less toxic than the all-L peptide against mammalian cells

Esc(1-21) and its diastereomer were analyzed for their effect on the viability of human type II alveolar epithelial cell line (A549 cells) by the MTT assay. They did not significantly reduce the percentage of viable cells at concentrations up to 64 μM (Fig. 1a). The LD_{50} values of the

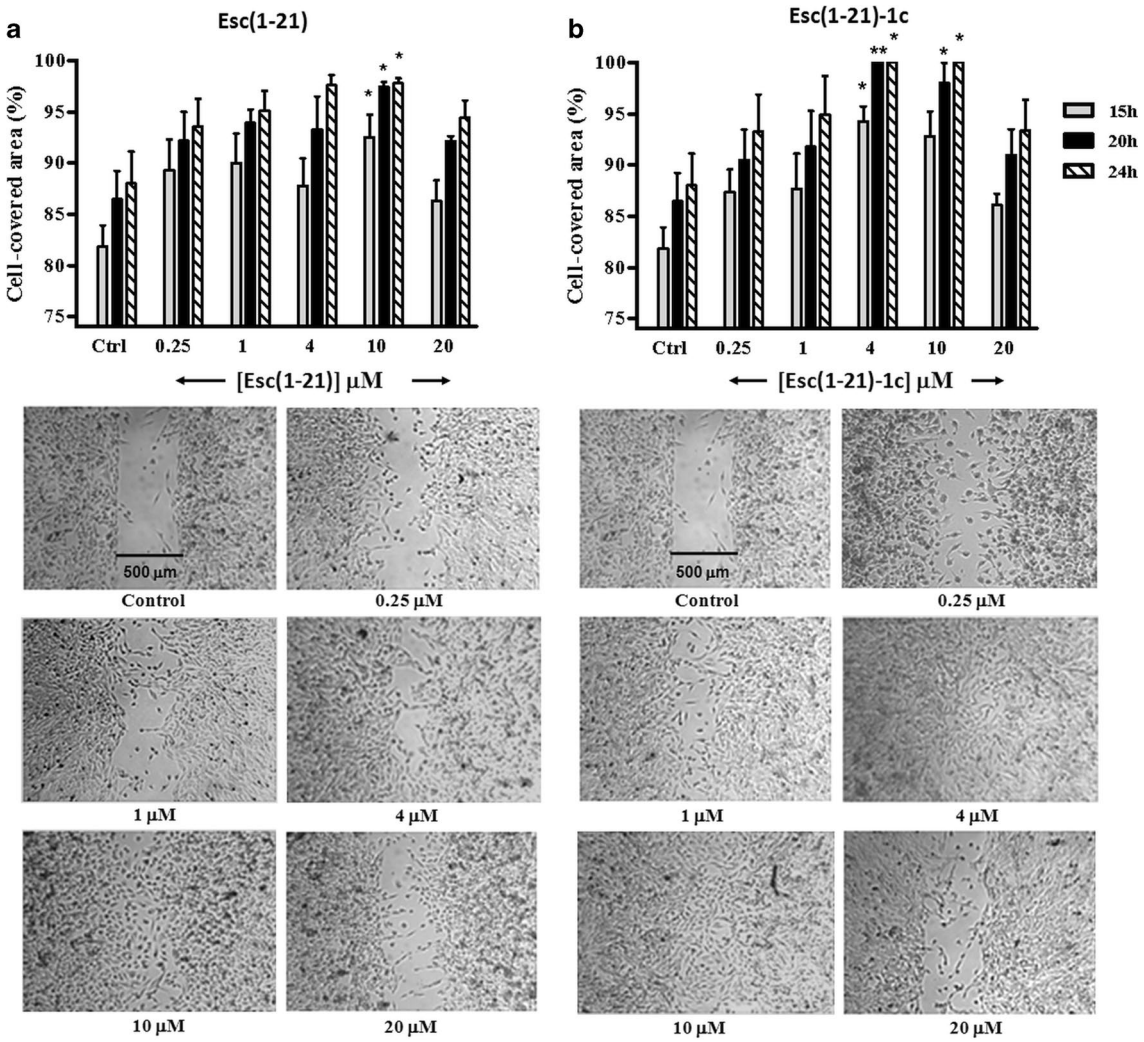

Fig. 2 Effect of Esc(1-21) (*panel a*) and its diastereomer Esc(1-21)-1c (*panel b*) on the closure of a pseudo-"wound" field produced in a monolayer of lung epithelial cells. A549 cells were seeded in each side of an ibidi culture insert and grown to confluence. Afterwards, they were treated or not with the peptide at different concentrations, as indicated. Cells were photographed at the time of insert removal (0 h) and examined for cell migration after 15, 20 and 24 h from peptide addition. The percentage of cell-covered area at each time point is reported on the *y-axis*. Control (Ctrl) are cells not treated with the peptide. All data are the mean of at least three independent experiments ± SEM. The levels of statistical significance between Ctrl and treated samples are indicated as follows $*p < 0.05$, $**p < 0.01$. *Micrographs* show representative results of pseudo-"wound" closure induced after 24 h peptide treatment at different concentrations with respect to the Ctrl sample

peptides were 150 μM and >256 μM for the wild-type and the diastereomer Esc(1-21)-1c, respectively. This difference was even more pronounced against macrophages, since Esc(1-21) had an LD_{50} of ~64 μM and the diastereomer higher than 256 μM (Fig. 1b).

The diastereomeric peptide is more efficient in promoting migration of human lung epithelial cells

It is known that colonization of lung epithelia by *Pseudomonas* leads to tissue damage with impairment of lung functions (Baltimore et al. 1989). Taking into account that type II pulmonary epithelial cells are responsible for epithelial reparation upon injury (Chuquimia et al. 2013), we studied the peptides' capability to promote migration of A549 cells in an in vitro pseudo-"wound" healing assay, by means of special cell-culture inserts. Both peptides were able to stimulate the closure of a gap produced in a monolayer of A549 cells. The wild-type peptide induced ~98 % coverage of the pseudo-"wound" field within 24 h at a concentration of 10 μM (Fig. 2a). In comparison, the diastereomer Esc(1-21)-1c led to the complete gap closure at 4 μM within 20 h (Fig. 2b).

We further investigated whether the peptide-induced cell migration involved an EGFR-mediated signaling

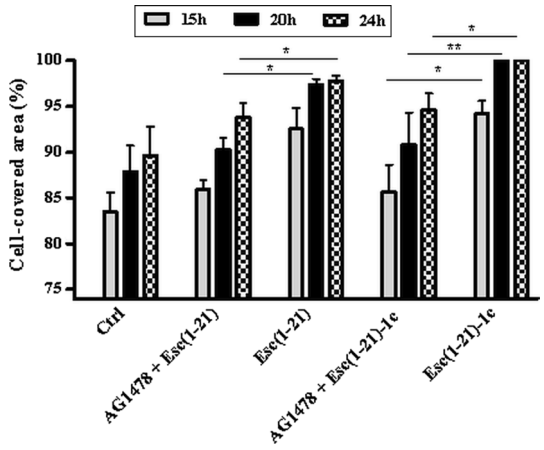

Fig. 3 Effect of AG1478 inhibitor on the peptide-mediated closure of a pseudo-"wound" field produced in a monolayer of A549 cells. After removal of the ibidi culture insert, A549 cells were pre-incubated with 0.2 μM AG1478 for 15 min and subsequently treated with 10 μM Esc(1-21) or 4 μM Esc(1-21)-1c. Some samples were treated with the peptide alone. Cells incubated with medium served as control (Ctrl). Samples were photographed at different time intervals, as indicated in the legend to Fig 2, and the percentage of cell-covered area was calculated and reported on the *y-axis*. All data are the mean of at least three independent experiments ± SEM. The levels of statistical significance between samples pre-treated with AG1478 and subsequently incubated with the peptide and those treated with the peptide alone at the corresponding time intervals are indicated as follows $*p < 0.05$, $**p < 0.01$

pathway, as already found for Esc(1-21) on the immortalized human keratinocytes (HaCaT cells) (Di Grazia et al. 2015). Figure 3 shows that pre-incubation of cells with AG1478, a specifically selective inhibitor of EGFR tyrosine kinase (Osherov and Levitzki 1994; Levitzki and Gazit 1995; Tokumaru et al. 2005; Hoq et al. 2011) resulted in the inhibition of the peptide-induced closure of the pseudo-"wounds". The percentages of cell-covered area in samples treated with the D-amino acids peptide alone were significantly higher than those found when cells were pretreated with AG1478 (Fig. 3), at all time intervals ($p < 0.05$, < 0.01 and < 0.05 after 15, 20 and 24 h, respectively). In the case of Esc(1-21), a significant difference was found only after 20- and 24-h treatment ($p < 0.05$) (Fig. 3). All together, these results clearly emphasize the better efficacy of the diastereomer in promoting the repair of the "wounded" cells monolayer compared to the all-L peptide (for which a higher concentration and a longer time was needed) and the involvement of EGFR in the peptide-induced migration of epithelial cells.

Next, to assess whether the peptide-induced cell migration was accompanied by morphological changes of A549 cells, we used fluorescence microscopy. Both untreated (control) and peptide-treated cells were stained with phalloidin and Hoechst for cytoskeletal and nuclei detection, respectively. Control A549 cells (Fig. 4a) as wells as those treated with 0.2 μM AG1478 (Fig. 4a') appeared organized into clusters with a regular morphological shape. Differently, cells treated either with Esc(1-21) or Esc(1-21)-1c appeared elongated, polarized and separated one from each other (Fig. 4b, b', respectively), which is consistent with an enhanced cell motility. However, these changes were not identified when the two peptides were added to A549 cells after AG1478 pretreatment (Fig. 4c, c'). These data support the participation of EGFR in the esculentin-elicited migration of alveolar epithelial cells (Fig. 3).

Moreover, to discriminate the contribution of cell migration and proliferation in the peptide-induced recovery of the integrity of A549 monolayers, the pseudo-"wound" healing assay was performed by pretreating cells with the cell proliferation blocker mitomycin C (Chieng-Yane et al. 2011; Wang et al. 2012). As can be seen in Fig. 5, cell proliferation was not essential for the re-epithelialization process, as pretreatment with mitomycin C did not bias the cell migration pattern stimulated by both peptides. Overall, besides the finding of more rapid and efficient re-epithelialization activity of Esc(1-21)-1c compared to the wild-type Esc(1-21), our data indicate that the pseudo-"wound" healing activity primarily depends on the peptide-induced migratory activity of A549 cells.

Both esculentin derivatives neutralize LPS and prevent TNF-α secretion from LPS-activated macrophages

To get insight into additional host-defense properties, we tested Esc(1-21) and its diastereomer for their ability to inhibit the secretion of the pro-inflammatory cytokine TNF-α from murine macrophages after stimulation with LPS (10 ng/ml) from *P. aeruginosa.* The results demonstrated a dose-dependent effect in the inhibition of TNF-α release; 80 and 90 % inhibition at 10 and 20 μM Esc(1-21), respectively, compared to that of LPS alone (Fig. 6). In comparison, a weaker activity was detected with the diastereomer Esc(1-21)-1c; 20 and 30 % inhibition at 10 and 20 μM, respectively (Fig. 6).

Esc(1-21)-1c has less helical content than the all-L peptide in different lipid environments

The secondary structure of the peptides was determined by using CD spectroscopy in several environments: Hepes buffer, LPC, which mimics the zwitterionic nature of the plasma membrane of eukaryotic cells, or LPS (Fig. 7). Both peptides formed β-sheet structure in 5 mM Hepes (aqueous solution), but changed their conformation in LPC and LPS. Whereas Esc(1-21) adopted predominantly α-helix in both LPC and LPS environments, the CD spectra of the diastereomer indicated a loss of most of the α-helical structure.

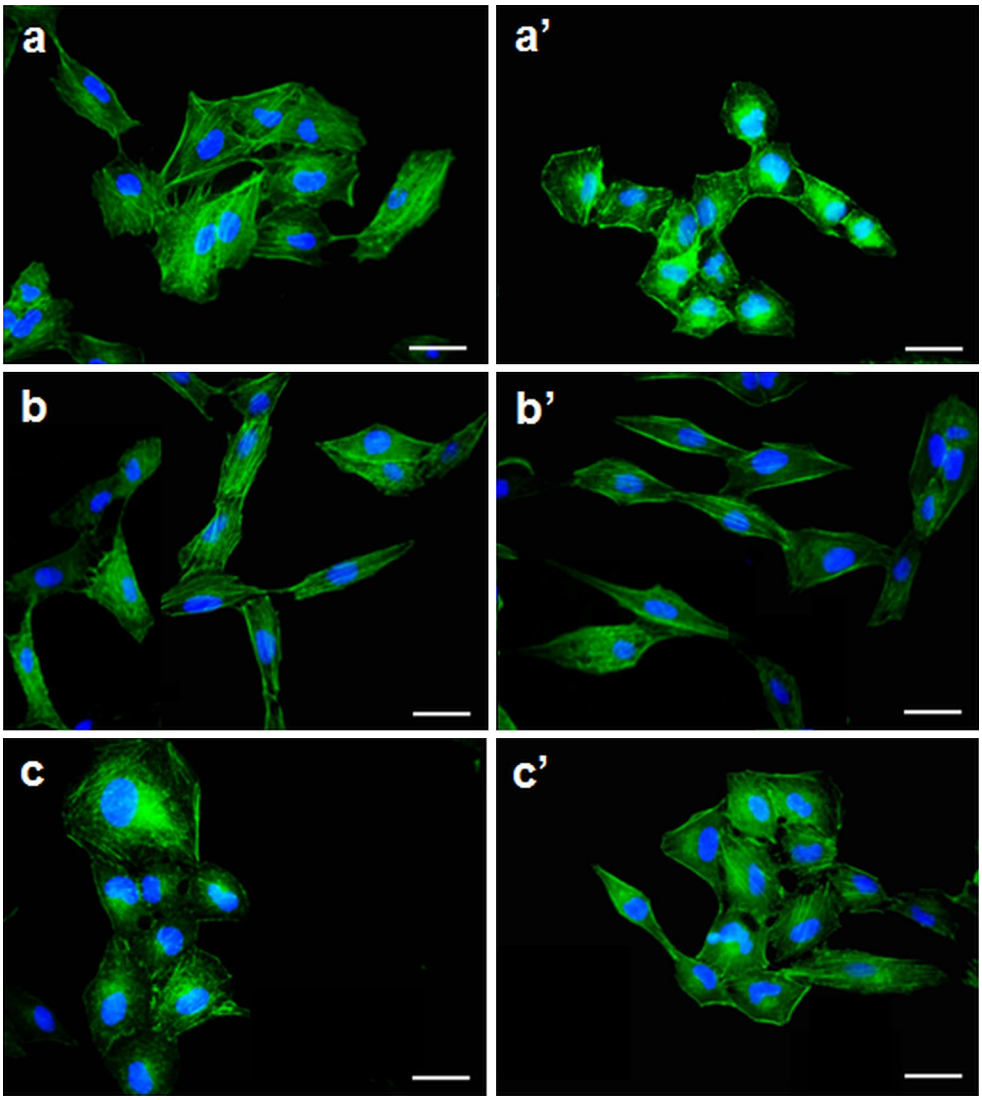

Fig. 4 Effects of Esc(1-21) and Esc(1-21)-1c on the morphology of A549 cells. After fixation in formaldehyde, cells were stained with Hoechst (for nuclei detection) and phalloidin (for cytoskeletal detection). The untreated control cells (**a**) as well as those treated with 0.2 μM AG1478 for 15 min (**a'**) appeared organized in clusters. The Esc(1-21) and Esc(1-21)-1c-treated cells (**b** and **b'** respectively) appeared elongated with a change in their shape and cytoplasmic protrusions. These alterations were not identified in A549 cells pretreated with AG1478 and subsequently exposed to Esc(1-21) and Esc(1-21)-1c (**c** and **c'**, respectively). *Bars* are 20 μm long

This could be the result of the incorporation of D-amino acids that (1) break an α-helical structure, and (2) induce an opposite CD spectra, because CD is chirality dependent.

Esculentin derivatives disassemble LPS aggregates

Light scattering experiments were used to figure out whether the peptides could alter the micelle morphology of LPS (above its critical micelle concentration). The data revealed that LPS from *Pseudomonas* is poly-dispersed in solution with major size-populations having hydrodynamic radius centered at 355 nm (Fig. 8). Incubation of LPS with Esc(1-21) caused a shift of the average size of LPS to a lower value centered at 67.7 nm, compared to the diastereomer Esc(1-21)-1c which induced a larger LPS mean diameter centered at 116, indicating weaker potency to dissociate LPS aggregates.

Discussion

Previously, we identified a short-length peptide, Esc-1a(1-21)NH_2, derived from the frog skin AMP esculentin-1a, with promising in vitro and in vivo anti-*Pseudomonal* activities (Luca et al. 2013). Here, we focused on the immunomodulating properties of this peptide and the effect of incorporation of D-amino acids on both its antimicrobial

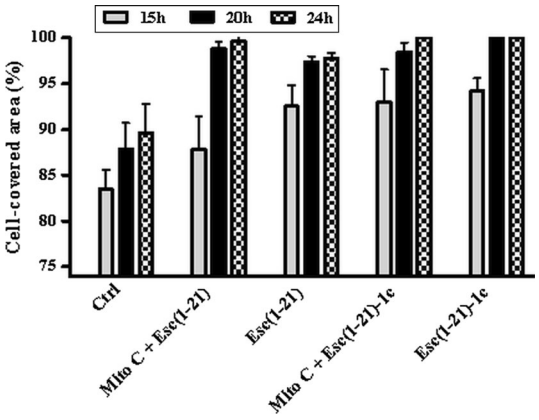

Fig. 5 Effect of mitomycin C on the peptide-mediated closure of a pseudo-"wound" field produced in a monolayer of A549 cells. After removal of the ibidi culture insert, A549 cells were pre-incubated with 5 μM mitomycin (Mito C) for 90 min and subsequently treated with 10 μM Esc(1-21) or 4 μM Esc(1-21)-1c. Some samples were treated with the peptide alone. Cells incubated with medium served as control (Ctrl). Samples were photographed at different time intervals, as indicated in the legend to Fig. 2, and the percentage of cell-covered area was calculated and reported on the *y-axis*. All data are the mean of three independent experiments ± SEM. No statistically significant difference was found between samples pretreated with Mito C and subsequently incubated with the peptide and those treated with the peptide alone, at all time intervals

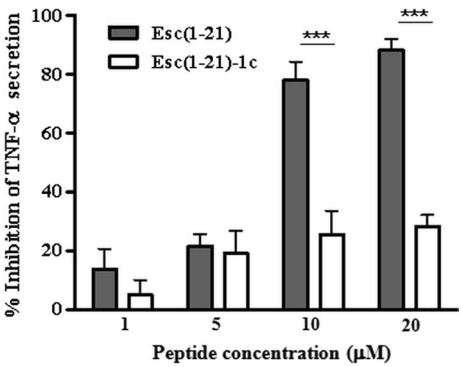

Fig. 6 The effect of peptides on the secretion of TNF-α from murine macrophages. RAW 264.7 macrophages were stimulated with LPS (10 ng/ml) derived from *P. aeruginosa* 10 in the presence of 1, 5, 10 and 20 μM Esc(1-21) or Esc(1-21)-1c for 4 h at 37 °C and 5 % CO_2. The percentage of inhibition of TNF-α release was normalized to that of macrophages stimulated with LPS without peptides (0 % inhibition). All the results are the mean of three independent experiments ± SEM. The level of statistical significance between samples treated with Esc(1-21) and Esc(1-21)-1c is indicated as follows ***$p < 0.001$

and non-antimicrobial activities. Functionally, the D-amino acids containing diastereomer Esc(1-21)-1c retains potent activity against the planktonic form of *P. aeruginosa* and it is more efficient than the all-L peptide against the sessile form of this bacterium, which is very difficult to eradicate. Furthermore, the diastereomer is significantly less toxic than Esc(1-21) towards both mammalian macrophages and alveolar epithelial cells. In addition, it is endowed with a higher stability in serum and with a higher activity in promoting migration of lung epithelial cells in an in vitro re-epithelialization assay. This suggests better propensity of the diastereomer Esc(1-21)-1c to heal damaged lung tissue. The enhanced stability in serum and lower toxicity to epithelial cells likely justify the diastereomer Esc(1-21)-1c to be a better candidate in future clinical application.

Mode-of-action studies pointed out that as found for Esc(1-21) on HaCaT cells, migration of alveolar epithelial cells induced by both isomers is prevented by AG1478, corroborating the involvement of EGFR in the signaling pathway controlling such an event. Similar results were also described for the frog skin AMPs temporins A and B (Di Grazia et al. 2014) and the cathelicidin LL-37 towards keratinocytes (Tokumaru et al. 2005) or corneal epithelial cells (Huang et al. 2007). It is known that plasma membrane-associated tyrosine kinase receptors, including EGFR, are localized in ordered lipid domain called lipid rafts (Pike et al. 2005) whose alteration may inhibit ligands binding to EGFR and its activation. Due to insertion of α-helical peptides in zwitterionic phospholipid bilayers (see below), the difference in wound healing activity between Esc(1-21) and Esc(1-21)-1c may be explained on the basis of a more pronounced change in the plasma membrane phospholipid organization and a resulting weaker activation of EGFR by the more helical Esc(1-21) compared to its diastereomer.

Importantly, in line with what we lately observed for Esc(1-21) on HaCaT cells (Di Grazia et al. 2015), the gap closure driven by both esculentin isoforms does not appear to be affected by cell proliferation, but rather depends on the cell migration activity. Indeed, cells exposed to mitomycin C before peptide treatment gave the same results as those obtained when the peptide was used alone (Fig. 5).

Esc(1-21)-1c has also the capability to inhibit the release of the pro-inflammatory cytokine TNF-α from macrophages activated by *P. aeruginosa* LPS, albeit to a lesser extent compared to the all-L parental peptide.

To define whether the secondary structure of the two peptides contributes to their different behavior in the toxicity studies and LPS detoxification activity, CD spectroscopy was carried out. We found that Esc(1-21) but not its diastereomer, folds into a predominantly α-helical structure in the presence of LPC. These results are consistent with the higher cytotoxicity of the wild-type Esc(1-21) compared to Esc(1-21)-1c. Indeed, numerous studies conducted with various native AMPs emphasized the importance of an amphiphatic α-helical structure for mammalian cell lysis (Pouny et al. 1992; Gazit et al. 1994; Strahilevitz et al. 1994).

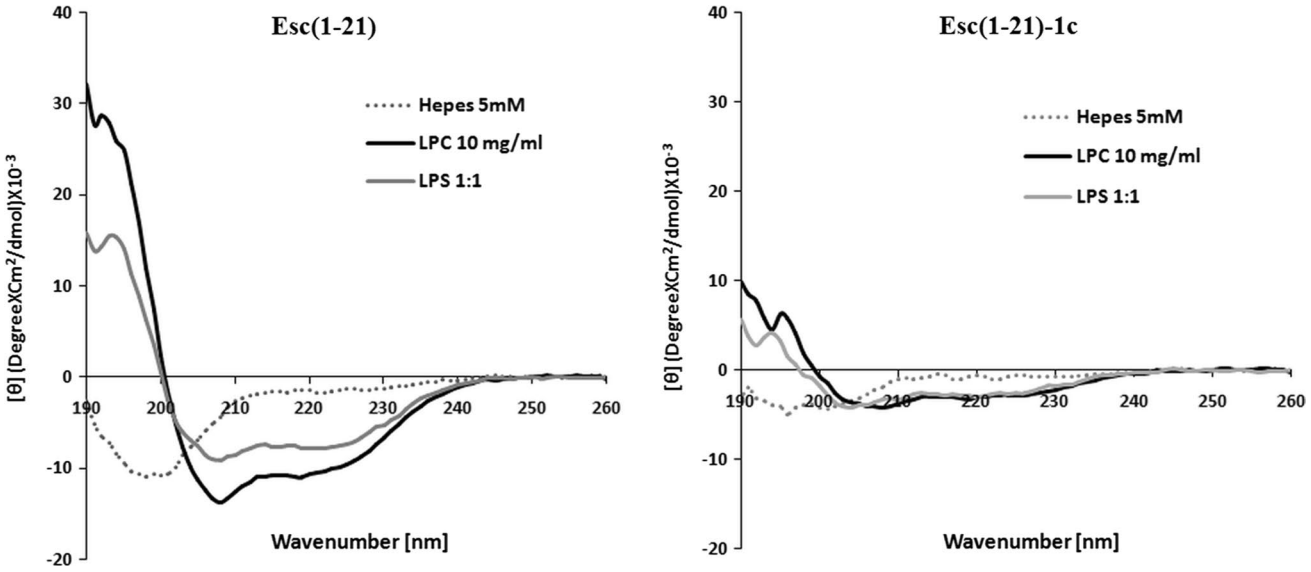

Fig. 7 The effect of LPS and LPC on the structure of Esc(1-21) or Esc(1-21)-1c. Circular dichroism spectra of 50 μM peptide in 5 mM Hepes (*dotted line*), 50 μM of purified *P. aeruginosa* LPS (*grey line*) or 10 mg/ml LPC (*black line*)

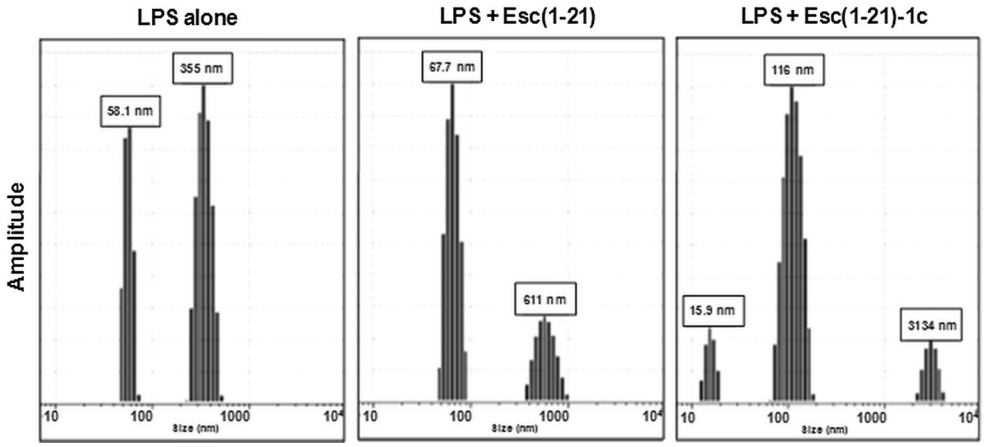

Fig. 8 The effect of peptides on the structural organization of LPS micelles. Light scattering of LPS from *P. aeruginosa* 10 in water before and after incubation with Esc(1-21) or Esc(1-21)-1c at equimolar concentration

This is likely assisted by a deeper insertion of peptides with a higher helical content into the hydrophobic core of mammalian cell membranes (Carotenuto et al. 2008; Mangoni et al. 2011). Furthermore, our findings are in line with previous reports showing that incorporation of D-amino acids into native AMPs, such as the cytolytic peptides pardaxin (Shai and Oren 1996) and melittin (Oren and Shai 1997) or synthetic peptides causes (1) abrogation of their toxic effect on mammalian cells while retaining antibacterial activity and ability to permeate anionic membranes (Oren et al. 1997), and (2) increases the resistance of diastereomers to serum inactivation (Oren et al. 1997).

Remarkably, LPS covers more than 90 % of the outer membrane in gram-negative bacteria and forms an impermeable barrier preventing the passage of both hydrophobic and hydrophilic molecules (Bhunia et al. 2011; Ghosh et al. 2014). Nevertheless, beside protecting bacteria from their surroundings, LPS can also act as an effector molecule by activating the host's immune response against invading microbial pathogens. Indeed, upon being shed from bacteria as a result of their death or cell division, it forms micelles, which are the active form of the endotoxin (Takayama et al. 1994; Rosenfeld and Shai 2006). However, when LPS is transferred to CD14, e.g., the primary receptor of LPS, which is mainly expressed on macrophages (Schumann et al. 1990), it disaggregates (Tobias and Ulevitch 1993). The LPS-CD14 complex then interacts with the transmembrane protein Toll-like receptor-4 (TLR4) and initiates the intracellular signaling cascade controlling the expression of cytokines, i.e., TNF-α (Rosenfeld et al. 2006b). The possible mechanism of action in which AMPs neutralize LPS by breaking its large micelles into smaller ones has been

proposed by others as well (Rosenfeld et al. 2006a, b; Bhunia et al. 2011). The disaggregated form of LPS can then interfere with its binding to the LPS-binding protein. As a consequence, TLR4 will not be activated and the synthesis of TNF-α will be prevented (Rosenfeld et al. 2006a, b; Mangoni and Shai 2011). Our light scattering analysis highlights the formation of smaller-sized LPS micelles upon addition of the peptides, with a stronger effect in the presence of the all-L Esc(1-21).

Note that peptides characterized by having a well-defined and stabilized structure in both LPS and cytoplasmic membrane and by the capability to disassemble the structural organization of LPS micelles are known to have a potent LPS detoxification activity (Rosenfeld et al. 2008). Therefore, the higher α-helical content of Esc(1-21) in LPS and its stronger efficacy in disrupting LPS aggregates compared to its diastereomer are consistent with its stronger anti-endotoxin activity (Fig. 6). Importantly, by suppressing the secretion of pro-inflammatory cytokines from activated immune cells, these peptides would contribute to a down-regulation of acute inflammatory responses elicited during infection or injury; an advanced benefit of an antimicrobial agent in vivo. The mild anti-inflammatory activity of the diastereomer compared to the wild-type peptide might be an advantage when the host's innate immune response to bacterial infection is at the beginning stage.

Conclusions

In conclusion, we have demonstrated multiple functions for both the wild-type peptide and its diastereomer. Importantly, only two L-to-D-amino acids substitutions in the C-terminal end of Esc(1-21) are sufficient to: (1) significantly reduce its cytotoxic effect towards mammalian cells (e.g., alveolar epithelial cells and macrophages); (2) increase its effectiveness against the biofilm form of *P. aeruginosa*, while maintaining a high activity against the free-living form of this important pathogen; (3) enhance the peptide's biostability; and (4) improve the ability of the peptide to promote migration of lung epithelial cells and presumably its capacity to restore the integrity of the injured lung tissue. To the best of our knowledge, this is the first report showing multiple beneficial effects of D-amino acids incorporation into an AMP. These multiple beneficial properties make the diastereomer a better candidate for future medical preparations against *P. aeruginosa*-induced infections, particularly in CF patients, while offering important findings to assist and optimize the future design of new AMPs exhibiting multiple functions.

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Conflict of interest The authors declare that they have no conflict of interest.

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