Esculentin-1a-derived peptides promote clearance of pseudomonas aeruginosa internalized in bronchial cells of cystic fibrosis patients and lung cell migration: Biochemical properties and a plausible mode of action

Esculentin-1a-derived peptides promote clearance of *P. aeruginosa* internalized in cystic fibrosis bronchial cells as well as lung cells migration: Biochemical properties and a plausible mode of action

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ABSTRACT

Pseudomonas aeruginosa is the major microorganism colonizing the respiratory epithelium in cystic fibrosis (CF) sufferers. The widespread usage of available antibiotics has drastically reduced their efficacy, and antimicrobial peptides (AMPs) are a promising alternative. Among them, the frog-skin derived AMPs i.e. Esc(1-21) and its diastereomer Esc(1-21)-1c have recently shown potent activity against free-living and sessile forms of *P. aeruginosa.* Importantly, this pathogen also escapes antibiotics treatment by invading airway epithelial cells. Here we demonstrate that both AMPs kill *Pseudomonas* once internalized into bronchial cells which express either the functional or the ΔF508 mutant of CF transmembrane conductance regulator. A higher efficacy is displayed by Esc(1-21)-1c (90% killing at 15 µM in 1h). We also show the peptides' capability to stimulate migration of these cells and restore the induction of cell migration that is inhibited by *Pseudomonas* lipopolysaccharide when used at concentrations mimicking lung infection. This property of AMPs was not investigated before. Our findings suggest new therapeutics that not only eliminate bacteria but also can promote re-epithelialization of the injured infected tissue. Confocal microscopy indicated that both peptides are intracellularly localized with a different distribution. Biochemical analyses highlighted that Esc(1-21)-1c is significantly more resistant than the all-L peptide to bacterial and human elastase, which is abundant in CF lungs. Beside proposing a plausible mechanism underlying the properties of the two AMPs, the data are discussed with regards to differences between them and suggest Esc(1-21)-1c as a candidate for the development of a new multifunctional drug against *Pseudomonas* respiratory infections.

Keywords: frog skin-antimicrobial peptide; *Pseudomonas aeruginosa*; bronchial cells; wound healing; lipopolysaccharide; elastase

INTRODUCTION

Pseudomonas aeruginosa is an opportunistic Gram-negative bacterium characterized by an intrinsic high resistance to commonly used antimicrobials (1, 2) and by its ability to form sessile communities, named biofilms (3-5). In this scenario, *P. aeruginosa* infections can easily take over and affect multiple organ systems such as the respiratory tract, particularly in cystic fibrosis (CF) patients (6-8). The most common mutation associated with CF phenotype is phenylalanine deletion at position 508 (ΔF508) in the CF transmembrane conductance regulator (CFTR) gene (9) encoding an ABC transporter that functions as a chloride channel in the membrane of epithelial cells (10). As a result of this mutation, the secretion of chloride ions outside the cell is inhibited, resulting in the generation of a dehydrated and sticky mucus layer coating the airway epithelia (11, 12). This helps the accumulation of trapped microbes, including *P. aeruginosa*, with deterioration of lung tissue and impairment of respiratory functions (13-15).

Importantly, *P. aeruginosa* colonization of host tissues is triggered by an initial attachment of the bacterium to epithelial cells (7, 16), via a variety of surface appendages (e.g. flagella, pili) (17-19). This is then followed by cell internalization, presumably mediated by binding of the bacterial lipopolysaccharide (LPS, i.e. the major component of the outer membrane in Gram-negative bacteria) to CFTR (20-24). Other mechanisms include e.g. interaction with asialoganglioside 1 (25). Invasion of host cells is a common process used by different microbial pathogens to facilitate escape from immune factors and/or to assist systemic diffusion and infection (26, 27). Intracellular persistence of bacteria that spread into the respiratory tract of CF patients may be one of the reasons

69 responsible for the chronic nature of *P. aeruginosa* lung infections (17). It protects the bacteria from
70 the host defense mechanisms and from the killing action of conventional antibiotics that hardly
71 enter epithelial cells (28). Hence, the discovery of new antibiotics with new modes of action is
72 highly demanding, and naturally occurring antimicrobial peptides (AMPs) represent potential
73 alternatives (29, 30). AMPs are produced by all living organisms as the first barrier against invading
74 microorganisms (31) and the majority of them are characterized by having a net positive charge at
75 neutral pH and the tendency to form an amphipathic structure in a hydrophobic environment (32,
76 33).

77 Recently, we studied a derivative of the frog skin AMP esculentin-1a, Esc-1a(1-21)NH_2 [(Esc(1-21)
78 GIFSKLAGKKIKNLLISGLKG-NH_2] (34, 35), corresponding to the first 20 residues of esculentin-
79 1a, as well as its diastereomer Esc(1-21)-1c containing two D-amino acids at positions 14 and 17
80 (i.e. D-Leu and D-Ser, respectively). The data revealed that the two peptides have a strong
81 bactericidal activity against both the planktonic and biofilm forms of *P. aeruginosa*, with the
82 diastereomer being more active than the wild-type peptide on the sessile form of this pathogen (36).
83 Furthermore, the diastereomer is more stable in human serum (36). However, there are no studies
84 on the effect of these peptides on infected lung epithelial cells, and only limited information is
85 available for other AMPs.
86 Importantly, the ability of a peptide to restore the integrity of a damaged infected tissue for example
87 by accelerating migration of epithelial cells in addition to a potent antimicrobial activity, would
88 make it a more promising candidate for the development of a new anti-infective agent. Another
89 advantage is the ability to resist degradation by proteases. Here we report on the effect of the two
90 esculentin-derived AMPs on the viability of bronchial epithelial cells expressing a functional CFTR
91 or a mutant form of CFTR (ΔF508-CFTR). Furthermore, we investigated the peptides' ability (i) to

kill a clinical isolate of *P. aeruginosa*, once internalized in the two bronchial cell lines, and (ii) to stimulate migration of bronchial cells in the presence of concentrations of LPS that better simulate an infection condition, by means of a pseudo-"wound" healing assay. To the best of our knowledge this is the first demonstration of cell migration induced by AMPs in the presence of LPS. In addition, we studied the peptides' distribution within bronchial cells and their stability to elastase from *P. aeruginosa* and human neutrophils. The data is discussed with regards to the different biochemical properties of the two peptides, and a plausible mechanism for their antimicrobial and wound healing properties is proposed.

MATERIALS AND METHODS

Materials. Minimal Essential Medium (MEM), heat-inactivated fetal bovine serum (FBS), penicillin/streptomycin were from Euroclone (Milan, Italy); puromycin, gentamycin, 3(4,5-dimethylthiazol-2yl)2,5-diphenyltetrazolium bromide (MTT), Triton X-100, AG1478, 4',6-diamidino-2-phenylindole (DAPI), Rhodamine, Mowiol 4-88, LPS from *P. aeruginosa* serotype 10 (purified by phenol extraction) and elastase from human leukocytes were purchased from Sigma-Aldrich (St. Luis, MO). Elastase from *P. aeruginosa* was from Millipore Merck (Merck, Mi, Italy). All other chemicals were reagent grade.

Peptides synthesis. Synthetic Esc(1-21) and its diastereomer Esc(1-21)-1c as well as rhodamine-labeled peptides [rho-Esc(1-21) and rho-Esc(1-21)-1c] were purchased from Chematek Spa (Milan, Italy). Briefly, each peptide was assembled by step-wise solid-phase synthesis using a standard F-

moc strategy and purified via RP-HPLC to a purity of 98%, while the molecular mass was verified by mass spectrometry.

Cells and bacteria. The following cell cultures were employed: immortalized human bronchial epithelial cells derived from a CF patient (CFBE41o-), but transduced with a lentiviral system to stably express ΔF508-CFTR (ΔF508-CFBE) or functional CFTR (wt-CFBE) (37). Cells were cultured in MEM supplemented with 2 mM glutamine (MEMg) plus 10% FBS, antibiotics (0.1 mg/ml of penicillin and streptomycin) and puromycin (0.5 µg/ml or 2 µg/ml for wt-CFBE or ΔF508-CFBE, respectively) at 37 °C and 5% CO_2 in 75-cm^2 flasks. The following bacterial strain was used: the invasive clinical isolate from the early stage of chronic lung infection, *P. aeruginosa* KK1, from collection of the CF clinic Medizinische Hochschule of Hannover, Germany (38, 39).

Peptides' effect on the viability of airway epithelial cells. The effect of both peptides on the viability of wt-CFBE or ΔF508-CFBE cells was evaluated by the MTT colorimetric method (40). MTT is a tetrazolium salt which is reduced to a colored formazan product by mitochondrial reductases in metabolically-active cells. Briefly, about $4x10^4$ cells suspended in MEMg supplemented with 2% FBS were seeded in wells of a 96-well microtiter plate. After overnight incubation at 37 °C and 5% CO_2 atmosphere, the medium was removed and 100 µl of fresh serum-free MEMg or 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) with or without the peptide at different concentrations were added to each well. After 2h or 24h, as indicated, at 37 °C in a 5% CO_2 atmosphere, the medium was replaced with 100 µl of Hank's buffer containing 0.5 mg/ml

MTT. The plate was incubated at 37 °C and 5% CO_2 for 4h and the formazan crystals were dissolved by adding 100 µl of acidified isopropanol according to (41). Absorption of each well was measured using a microplate reader (Infinite M200; Tecan, Salzburg, Austria) at 570 nm. The percentage of metabolically-active cells compared to control samples (cells not treated with peptide) was calculated according to the formula:

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

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

Cell infection and peptide's effect on intracellular bacteria. About 100,000 bronchial cells in MEMg supplemented with 10% FBS were seeded in 24-well plates and grown for two days at 37 °C and 5% CO_2. The clinical isolate KK1 was grown in Luria-Bertani broth at 37 °C with mild shaking (125 rpm) to mid-log phase (optical density of 0.8 at 590 nm), and subsequently harvested by centrifugation. The pellet was then resuspended in MEMg and properly syringed using a 21 gauge needle to avoid clumps formation before infecting cells. A multiplicity of infection (MOI) of 100: 1 (bacteria:cells) was used: 200 µl of this bacterial suspension containing about $1x10^7$ colony forming units (CFU) was co-incubated for 1h with wt-CFBE/ΔF508-CFBE at 37 °C and 5% CO_2. After infection, the medium was removed and the cells were washed three times with MEMg and then incubated for 1h with a gentamycin solution (200 µg/ml in MEMg) to remove extracellular bacteria. Afterwards, the medium was aspirated and the infected cells were washed three times, as described above. Two hundred microliters of Hank's solution with or without the peptide at different concentration was added to each well and the plate was incubated for 1h at 37 °C and 5%

158 CO_2. After peptide treatment, cells were washed with phosphate buffered saline (PBS) and lysed
159 with 300 µl of 0.1% Triton X-100 in PBS for 15 min at 37 °C and 5% CO_2. Each sample was then
160 sonicated in a water bath for 5 min to break up possible bacterial clumps, and appropriate aliquots
161 were plated on agar plates for counting of CFU after 24h at 37 °C.

162

163 ***In vitro* cell migration assay.** The ability of single peptides or *P. aeruginosa* LPS to stimulate
164 migration of CF cells was evaluated by a modified scratch assay, as reported in (36). Briefly,
165 special cell culture inserts for live cell analysis (Ibidi, Munich, Germany) were placed into wells of
166 a 12-wells plate. About 35,000 cells suspended in MEMg supplemented with 10% FBS were seeded
167 in each compartment of the culture insert and incubated at 37 °C and 5% CO_2 for approximately
168 24h to allow cells to grow to confluence. Afterwards, inserts were removed to create a cell-free area
169 (pseudo-"wound") of approximately 500 µm; 1 ml MEMg with or without the peptide or LPS at
170 different concentration was added to each well. Plates were incubated as above to allow cells to
171 migrate and samples were visualized at different time intervals under an inverted microscope
172 (Olympus CKX41) at x 4 magnification and photographed with a Color View II digital camera. The
173 percentage of cell-covered area at each time was determined by WIMASIS Image Analysis
174 program.

175 In another set of experiments, cells migration was evaluated in the presence of both LPS and Esc(1-
176 21) or Esc(1-21)-1c at the indicated concentration. Furthermore, the implication of epidermal
177 growth factor receptor (EGFR) in peptide-induced cell migration was analyzed by pretreating cells
178 for 30 min with 5 µM of tyrosine phosphorylation inhibitor tyrphostin (AG1478) (42).

179

Fluorescence Studies. About $2x10^5$ bronchial cells in MEMg supplemented with 10% FBS were seeded on 0.13-0.17 mm thick coverslips (properly put into 35 mm dish plates) and incubated at 37 °C and 5% CO_2. After 24h, samples were washed with 1 ml PBS and treated with 4 µM rhodamine-labelled peptide or rhodamine (for control samples) in MEMg. After 30 min and 24h incubation at 37 °C and 5% CO_2, cells were washed four times with PBS and fixed with 700 µl of 4% formaldehyde for 15 min at room temperature. Afterwards, they were washed twice with PBS and stained with DAPI (1 µg/ml) for 5 min at room temperature to visualize the nuclei. After three additional washes, the coverslips were mounted on clean glass slides using Mowiol mounting medium, and observed under a confocal microscope Olympus FV1000, objective lens 60X (oil). Data analysis was done using the Olympus Fluoview (Ver. 4.1) and Image J. Results are reported as the ratio between the fluorescence intensity of rhodamine-labeled peptides in the cytoplasm *versus* the nucleus.

Peptides' stability to proteases. Peptides were dissolved in 10 mM Tris-HCl pH 7.5 at a final concentration of 1 mg/ml; afterwards, 130 µl were incubated with 4 µg of human or bacterial elastase (final enzyme concentration equal to 1 µM). At indicated time intervals, 30 µl aliquots were withdrawn, diluted with 770 µl of 0.1% trifluoroacetic acid (TFA)/water and analyzed by RP-HPLC and mass spectrometry. Liquid chromatography was performed on Phenomenex Jupiter C18 analytical column (300 A°, 5 µm, 250 x 4.6 mm) in a 30 min gradient, using 0.1% TFA in water as solvent A and methanol as solvent B. Mass spectrometry analysis was performed with a Bruker Daltonic ultraflex MALDI TOF/TOF mass spectrometer on withdrawn samples as well as on HPLC-eluted peaks.

Statistical analyses. Quantitative data, collected from independent experiments, were 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

Peptides' effect on the viability of lung epithelial cells. In a previous study (36) it was reported that both Esc(1-21) and its diastereomer Esc(1-21)-1c are not toxic to A549 epithelial cells up to 64 µM, and that the diastereomer is substantially less toxic when used at higher concentrations (36). Although A549 cells possess morphological and biochemical properties of alveolar epithelial cells (type II pneumocytes) (17), they do not express CFTR (43, 44). We therefore studied the effect of both esculentin peptides on the viability of airway epithelial cells stably expressing the most common CFTR mutation (ΔF508) and the corresponding wild-type cell line expressing a functional CFTR.

As indicated by the results of the viability assay performed in the cell culture medium, MEMg (Fig.1), the two peptides were not toxic to both types of epithelial cells at the concentration range between 2 µM and 64 µM, within 2h. However, the wild-type peptide turned out to be toxic at 128 µM causing approximately 25% and 70% reduction in the percentage of metabolically-active wt-CFBE and ΔF508-CFBE cells, respectively (Fig.1A, B). Furthermore, after a long-term treatment (24h), the cytotoxicity of Esc(1-21) became more pronounced, inducing about 20-25% reduction of metabolically-active epithelial cells at 32 µM and 64 µM (Fig. 1C, D). A higher cytotoxicity was recorded at 128 µM with ~60% and 90% killing of wt-CFBE and ΔF508-CFBE cells, respectively. Importantly, the diastereomer did not induce any reduction in the percentage of metabolically-active cells (Fig. 1).

Peptides' activity on bronchial cells infected by the CF clinical isolate KK1. We monitored the effect of the two peptides on wt-CFBE (Fig. 2A and B) and ΔF508-CFBE cells (Fig. 2C and D) after infection with a clinical isolate of *P. aeruginosa* (KK1 strain). This strain was already shown to invade bronchial cells *in vitro* (38). According to the published literature (25), *P. aeruginosa* tends to target particular CF epithelial cells rather than making a concerted attack on the whole population of cells. Figure 2 shows the number of intracellular bacteria that were calculated either as CFU per sample (panels A and C) or as percentage (panels B and D) with respect to the peptide-untreated infected cells (control). Note that the number of internalized bacteria (~3,500 CFU) in our control bronchial cells (Fig. 2A and C) was well correlated to the previously found uptake of different *P. aeruginosa* strains by CF airway epithelial cells (21).

The data shown in Fig. 2B reveal that the two peptides, at 5 μM, caused about 15-20% killing of intracellular bacteria 1h after treatment. This is when they were used on wt-CFBE cells in Hank's buffer. This condition was used to better simulate a physiological environment without the presence of cell culture medium components. However, the killing effect was more evident in ΔF508-CFBE (Fig. 2D), where 5 μM Esc(1-21) and its diastereomer caused ~40% and 60% decrease in the number of intracellular bacteria, respectively, compared to untreated infected cells. Due to the negligible cytotoxicity of the diastereomer Esc(1-21)-1c compared to the all-L isomer in Hank's buffer (Fig. S1), it was possible to use Esc(1-21)-1c at higher concentrations without damaging host epithelial cells. Interestingly, 10 μM and 15 μM Esc(1-21)-1c gave rise to about 70% and 90% reduction in the survival of intracellular bacteria in ΔF508-CFBE (Fig. 2D), whereas only ~50% bacterial killing in wt-CFBE cells was obtained after exposure to 15 μM Esc(1-21)-1c (Fig. 2B). Note that the toxicity of Esc(1-21) to bronchial cells when tested in Hank's buffer, compared to

MEMg (Fig. 1), could be due to the absence of medium components that may affect its activity resulting in lower cytotoxicity (Fig. 1).

"Wound" healing assay in the presence of peptides, LPS or their combination. Since airway epithelium and CFTR have been shown to play a crucial role in maintaining lung function and wound repair (45), we investigated the effect of the two peptides on the migratory activity of the bronchial cells in the cell culture medium, MEMg. Both AMPs were able to stimulate migration of wt-CFBE and ΔF508-CFBE cells as indicated by their ability to induce ~100% coverage of the pseudo-"wound" field produced in the cell monolayer (by means of special cell culture inserts) within 20h at optimal concentrations of 10 µM for Esc(1-21) (Fig. 3A and B), or 1 µM for its diastereomer (Fig. 3C and D). A slower cell migration speed was observed for the mutant ΔF508-CFBE, in agreement with a previous study (45) that showed how a defective function of CFTR caused a reduced lamellipodia area from the leading edge of airways epithelial cells (46).

It is well known that following bacterial death or division, LPS (the major component of the outer membrane of Gram-negative bacteria) is released from the bacterial cell wall (6, 47). Recently, as described in (48), it was demonstrated that the presence of non-toxic levels of *P. aeruginosa* LPS accelerates wound repair in airway epithelial cells. We therefore analyzed the effect of different concentrations of *P. aeruginosa* LPS on the migratory activity of both wt-CFBE and ΔF508-CFBE. As illustrated in Figs. 4A and 4B, LPS promoted within 20h the closure of a 500 µm wide gap created in a monolayer of wt-CFBE or ΔF508-CFBE cells at optimal dosages of 1,000 ng/ml or in the range 1,000-10,000 ng/ml, respectively. Alternately, when LPS was used at higher concentrations (i.e. ≥ 15,000 ng/ml) that can be found in the sputum of CF patients with chronic *P. aeruginosa* lung infection (49), the coverage of the "wounded" area was significantly slackened

(Fig. 4A and B). To verify whether this LPS concentration was lethal to the cells, a viability assay was carried out. However, no cell damage was observed up to 20 µg/ml LPS, as indicated by the percentage of metabolically-active cells which was comparable to that of control samples (data not shown). This correlates with the negligible toxicity already found for LPS on both normal human bronchial epithelial cells (48) and A549 cells (50).

Interestingly, the induction of cells migration was clearly restored (Fig. 4C-F) when the LPS dosage that blocks the closure of the "wounded" field was used in combination with the optimal "wound" healing concentration of Esc(1-21)-1c (1 µM). A similar effect was noted when the inhibitory concentration of LPS was mixed with a concentration of Esc(1-21) lower than its optimal dosage in promoting migration of wt-CFBE and ΔF508-CFBE cells (4 µM and 1 µM, respectively, Fig. 4C-F).

Mechanism of peptide-induced cell migration. The airways of healthy or CF human subjects express EGFR (42), which is involved in the repair of damaged airways epithelium (51). Hence, we examined whether the peptide-promoted cell migration was a process mediated by EGFR-signaling pathway. For that purpose, we pretreated wt-CFBE cells with 5 µM AG1478, a selective inhibitor of EGFR tyrosine kinase (52), before adding each of the two peptides at their optimal concentrations in stimulating cell migration (10 µM and 1 µM for Esc(1-21) and Esc(1-21)-1c, respectively). A clear inhibition of pseudo-"wound" closure was observed, as was shown by the significantly lower percentages of cell-covered area at all-time intervals (15h, 20h, 24h), with respect to the results found when the bronchial cells were not pre-incubated with the inhibitor (Fig. 5). These results highlight the involvement of EGFR in the peptide-induced migration of bronchial cells. Similar results were obtained with the mutant ΔF508-CFBE (data not shown).

295

296 **Peptide distribution within bronchial cells.** In order to know the peptides' distribution in wt-
297 CFBE and ΔF508-CFBE cells, we used confocal microscopy and rhodamine-labeled peptides. As
298 reported in Fig. 6A and B, rho-Esc(1-21) was mainly aligned to the perinuclear region of the cell
299 already after 30 min from its addition, as well as after 24h. To address the possibility that
300 rhodamine facilitated the uptake of the peptide, we also used the rhodamine dye non-conjugated to
301 the peptide. However, rhodamine was not observed in the bronchial cells, as revealed by the lack of
302 fluorescence inside them (Fig. 6C, D). In comparison, in the case of rho-Esc(1-21)-1c, the
303 fluorescence intensity appeared to be evenly distributed within the cytosol and nucleus (Fig. 6E, F).
304 These findings were corroborated by the quantitative analysis of fluorescence intensity of the two
305 peptides between the cytoplasm and nucleus in both types of cells (Fig. 7).

306

307 **Peptides' susceptibility to human and *P. aeruginosa* elastase.** One of the main drawbacks in
308 using AMPs for treatment of *P. aeruginosa* lung infection is their susceptibility to enzymatic
309 degradation (53, 54). In particular, the lung environment of CF patients is rich in proteases, i.e.
310 elastase from host neutrophils and also from *P. aeruginosa* (55-57). Therefore, the stability of the
311 two peptides in the presence of both enzymes was studied. When elastase from human leukocytes
312 was used, Esc(1-21) was completely degraded within 5h (Table 1 and Fig. S2). In contrast, the
313 diastereomer was highly stable with 78% and 13% amount of non-degraded peptide after 5h and
314 24h incubation, respectively (Table 1). Mass spectrometry analysis confirmed the presence of one
315 main peak at 2,185 Da corresponding to the calculated molecular mass of Esc(1-21)-1c (Fig. S2). In
316 comparison, when the human cathelicidin AMP LL-37 was used as a reference, 44% of the peptide
317 remained after 5h treatment, but nothing was found after 24h (Table 1).

When *P. aeruginosa* elastase was used, both LL-37 and Esc(1-21) were completely degraded within 5h (Table 1), while in the case of Esc(1-21)-1c about 91% and 77% of non-degraded peptide was detected after 5h and 24h, respectively (33% after 48 h, data not shown).

Interestingly, mass spectrometry analysis of the samples revealed the presence of peaks with different molecular masses than those found after treatment with human elastase (Fig. S3), indicating different cleavage sites by the two elastases either in the two esculentin isoforms (Fig. S2 and S3) or in LL-37 (Fig. S4 and S5). Importantly, the main cleavage sites by human and bacterial elastase in Esc(1-21) were between Ile16 and Ser17 or between Asn13 and Leu14, respectively. Note that these two peptide bonds flank each of the two D-amino acids present in Esc(1-21)-1c (i.e. D-Leu14 and D-Ser17), thus preventing their proteolytic cleavage by both enzymes, compared to the all-L peptide.

DISCUSSION

The airway epithelial surface represents an important place where the host breaks off signals from inhaled microbial pathogens and activates defense mechanisms to combat infections, especially in chronic diseases. However, administration of exogenous molecules endowed with anti-infective and/or immunomodulatory properties are highly needed to speed up the recovery process (58). Nevertheless, treatment of respiratory infections has been compounded by the increasing resistance of pathogens to the commonly-used drugs. Thus, new candidates are urgently needed, among which AMPs represent a promising alternative. With respect to this, Esc(1-21) and primarily its diastereomer Esc(1-21)-1c have several advantages that support their development as new antipseudomonal agents (6). However, no studies were reported on the ability of these two peptides to clear intracellular *Pseudomonas* and only very limited information is documented for other

AMPs with reference to this matter. Furthermore, the ability of AMPs to stimulate migration of lung epithelial cells in the context of a bacterial infection (e.g. in the presence of pathogen associated molecular patterns, such as LPS) should be investigated in an attempt to develop a new drug that has also the capability to restore the normal architecture of the injured lung tissue. To the best of our knowledge, this has not yet been investigated for AMPs.

Here, we demonstrated the killing of intracellular *Pseudomonas* in bronchial cells with functional or mutated CFTR upon 1h exposure to the esculentin peptides. A significantly higher efficacy is displayed by the diastereomer Esc(1-21)-1c.

Note that conventional antibiotics (e.g. penicillins, cephalosporins and aminoglycosides) are not ideal choices for treating invasive infections due to their inability to penetrate the plasma membrane (59, 60). An exception is given by fluoroquinolones e.g. ciprofloxacin (61), which is currently used due to its strong activity against Gram-negative bacteria, in spite of its toxicity (62) and the increasing number of bacteria that are resistant to it (63). Remarkably, the diastereomer had a comparable activity to that of ciprofloxacin which was found to cause ~64%, 77% and 98% killing of intracellular bacteria when tested under our conditions on infected ΔF508-CFBE at 1 µM, 5 µM and 15 µM, respectively (data not shown).

By means of rhodamine-labeled peptides and confocal microscopy analysis we visualized an intracellular localization of the two molecules in which Esc(1-21) has a prevalent perinuclear distribution. No vesicular pattern of fluorescence was observed, excluding an endocytotic mechanism of peptide uptake which was shown in the case of Syn B peptides (64) and LL-37 in A549 cells after 30 min of incubation (65). A plausible explanation is that both esculentin isomers enter the cell by a self-translocation process through a peptide-induced membrane lipids destabilization without affecting cell viability (66). Indeed, when tested at non-cytotoxic

365 concentrations, the peptides were able to perturb the membranes of both wt-CFBE and ΔF508-
366 CFBE cells, as indicated by the increased fluorescence intensity of the small-sized membrane-
367 impermeable dye Sytox Green, upon peptides' addition to the cells (data not shown). This reflects
368 the intracellular influx of Sytox Green, presumably due to a mild membrane destabilization caused
369 by the peptides during their translocation across the cytoplasmic/nuclear membrane into the
370 cytosol/nucleus, respectively. Overall, we can assume that the clearance of intracellular
371 *Pseudomonas* is mainly due to the interaction of the internalized peptides with the bacterial cells. A
372 possible reason for the weaker efficacy of the all-L peptide Esc(1-21) to kill intracellular bacteria
373 compared to its diastereomer can be related to a stereochemical binding of Esc(1-21), but not Esc(1-
374 21)-1c, to intracellular components, making it less available. In addition, Esc(1-21) is expected to
375 be more susceptible to virulence factors, i.e. proteases released from the intracellular *P. aeruginosa*.
376 It is worth mentioning that the fluorescence intensity cannot tell us if the peptide is intact or
377 partially degraded or whether it is present in an inactive form. Furthermore, the intranuclear
378 location of the peptides would suggest additional mechanisms to boost their antimicrobial potency
379 e.g. by directly/indirectly upregulating the expression of genes involved in the host cell protection
380 from microbial pathogens.

381 Another important finding in this study is the pseudo-"wound" healing activity of the two
382 peptides in both wt-CFBE and ΔF508-CFBE cells. As reported for A549 cells (36), the
383 diastereomer is more effective in inducing re-epithelialization of the "wounded" area, with a
384 mechanism that implies EGFR-mediated signaling pathway. We think that the lower "wound"
385 healing efficacy of the more helical Esc(1-21) compared to its diastereomer is the consequence of a
386 stronger alteration of the phospholipid membrane region where EGF receptors are located. This
387 would impair the binding of ligands to EGFR with a resulting weaker EGFR activation (36).
388 However, the discrepancy between the two peptides in the "wound" healing activity may also be

related to a difference in the binding affinity of the two AMPs to the target receptor (presumably EGFR or membrane-bound metalloproteases (42, 66)). Furthermore, we cannot rule out the participation of alternative processes in controlling migration of bronchial cells. One of them could imply CFTR (especially in mutant cells), via yet to be discovered mechanism.

Here we also demonstrated for the first time that bronchial cells migration stimulated by both esculentin isomers is maintained in the presence of high concentrations of LPS, presumably found in CF sputum (49), and different plausible mechanisms underlying such events are discussed below. LPS has been found to regulate wound repair in airway epithelial cells through a signaling pathway triggered by its binding to the receptor TLR-4 (expressed on the airway epithelial cells) and implicating activation of EGFR (48, 67). Note that LPS binds TLR-4 in its monomeric form (68). However, in aqueous environments, micellar assemblies of LPS are formed above its critical micellar concentration values (≥14,000 ng/ml) (68, 69). This may hamper the binding of LPS to TLR-4, thus explaining the inhibitory pseudo-"wound" healing effect of LPS when used at high concentrations (i.e. ≥15,000 ng/ml, Fig. 4A and B). Note also that there is re-establishment of the migratory activity of bronchial cells when LPS (at its inhibitory concentration) is combined with Esc(1-21)-1c (at the optimal "wound" healing dosage, 1 µM, see Fig. 4C, D). This is presumably due to the diastereomer-induced re-epithelialization process, regardless of the presence of LPS. In contrast, a different mechanism would likely account for the increased pseudo-"wound" healing activity of Esc(1-21) when combined with high concentrations of LPS (Fig. 4C and D), compared to the results found when the peptide is used alone at the same concentrations (Fig. 3A and B). As previously reported, Esc(1-21) is more efficient than its diastereomer in disrupting LPS micelles (36). Such LPS disaggregation might be sufficient to reset the availability of LPS monomers for an optimal binding to TLR-4 and retrieval/improvement of "wound" healing activity.

Importantly, we also proved that the incorporation of two D-amino acids at specific positions in Esc(1-21) makes the peptide significantly more resistant to degradation by both human and bacterial elastases.

In conclusion, we have shown that: (i) A derivative of a frog-skin derived AMP and particularly its designed diastereomer rapidly kill *Pseudomonas* cells, once internalized in CF bronchial cells; (ii) The peptides accelerate bronchial cells migration under conditions that better simulate lung infection in CF (e.g. in the presence of LPS); (iii) The diastereomer has an overall higher efficacy than the all-L parent isoform. These findings are very important for the generation of new antimicrobial drugs that not only eliminate microbial pathogens, but would also recover the tissue integrity.

In this specific case, the aforementioned properties of Esc(1-21)-1c together with its higher biostability to elastases and undetectable cytotoxicity compared to the wild-type Esc(1-21) support further studies towards the development and usage of the former peptide for local treatment of *P. aeruginosa*-induced lung infections.

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629
630
631
632 **TABLE 1** Peptide amount after 5h and 24h incubation with human and *P. aeruginosa* elastase at 37
633 °C.
634

	Peptide remaining (%)[a]			
	Human elastase		*P. aeruginosa elastase*	
Peptide designation	**5h**	**24h**	**5h**	**24h**
Esc(1-21)	0	0	0	0
Esc(1-21)-1c	78	13	91	77
LL-37	44	0	0	0

635
636 [a]Peptide amounts were determined by the peak areas of the RP-HPLC relative to those of the
637 control peptide (dissolved in buffer) at 0 min (set as 100%)
638
639
640
641

642 **Figure legends**

643

644 **FIG. 1.** Peptides' effect on the viability of bronchial epithelial cells. About $4x10^4$ wt-CFBE (A, C)
645 or ΔF508-CFBE (B, D) cells were plated in wells of a microtiter plate. After overnight incubation at
646 37 °C in a 5% CO_2 atmosphere, the medium was replaced with 100 µl fresh MEMg supplemented
647 with the peptides at different concentrations. After 2h or 24h, cell viability was determined by the
648 MTT reduction to insoluble formazan. Cell viability is expressed as percentage with respect to the
649 control (cells not treated with the peptide). All data are the mean of four independent experiments ±
650 SEM. The levels of statistical significance between the two peptides are: *, $p< 0.05$; **, $p< 0.01$;
651 ****, $p< 0.0001$.

652

653 **FIG. 2.** Effect of Esc(1-21) and its diastereomer Esc(1-21)-1c on the intracellular killing of *P.*
654 *aeruginosa* KK1 in wt-CFBE (A, B) and ΔF508-CFBE (C, D) cells. About $1x10^5$ cells were seeded
655 in 24-well plates and grown to confluence. Afterwards, they were infected or not with the bacterium
656 for 1h; non-adherent extracellular bacteria were removed upon antibiotic treatment and infected
657 cells were treated or not for 1h with the peptide at different concentrations, as indicated. Control
658 samples (Ctrl) are peptide-untreated infected cells. The number of intracellular bacteria is expressed
659 either as CFU per sample (panels A and C) or as percentage with respect to the control (panels B
660 and D). All data are the mean of four independent experiments ± SEM. The levels of statistical
661 significance between peptides-treated infected cells and control samples are: *, $p< 0.05$; ***, $p<$
662 0.001; ****, $p< 0.0001$.

663

664 **FIG. 3.** Effect of Esc(1-21) and Esc(1-21)-1c on the closure of a pseudo-"wound" field produced in
665 a monolayer of wt-CFBE (A, C) and ΔF508-CFBE (B, D). Cells were seeded in each side of an
666 Ibidi culture insert and grown to confluence; afterwards, they were treated or not with the peptide.
667 Cells were photographed at the time of insert removal and examined for cell migration after 15, 20
668 and 24h from peptide addition. The percentage of cell-covered area at each time point is reported on
669 the y-axis. Peptide untreated cells were used as a control (Ctrl). The data are the mean of four
670 independent experiments ± SEM. The levels of statistical significance between Ctrl and treated
671 samples are: *, $p< 0.05$; **, $p< 0.01$; ***, $p< 0.001$; ****, $p< 0.0001$.

672

673 **FIG. 4.** Effect of *P. aeruginosa* LPS alone (A and B) or in combination with Esc(1-21)/Esc(1-21)-
674 1c (C-F) on the closure of a pseudo-"wound" field produced in a monolayer of wt-CFBE (A, C, E)
675 and ΔF508-CFBE (B, D, F). Cells were seeded in each side of an Ibidi culture insert and grown to
676 confluence; afterwards, they were treated or not with LPS or LPS + peptide. Cells were
677 photographed at the time of insert removal and examined for cell migration after 15, 20 and 24h
678 from addition of LPS or LPS + peptide. The percentage of cell-covered area at each time point is
679 reported on the y-axis. Control (Ctrl) used are cells not treated with LPS or peptide. All data are the
680 mean of four independent experiments ± SEM. The levels of statistical significance of samples
681 *versus* Ctrl (for panels A and B) or *versus* LPS-treated cells (for panels C and D) are: *, $p< 0.05$; **,
682 $p< 0.01$; ***, $p< 0.001$; ****, $p< 0.0001$. Micrographs showing representative results of wt-CFBE (E)
683 or ΔF508-CFBE (F) before (T0) and 24h after treatment (T24) with the combination LPS + peptide
684 (at the indicated concentrations in panels C or D) compared to samples treated with LPS are
685 reported. The black line marks the cell-free area in samples after 24h.

686

687 **FIG. 5.** The effect of AG1478 inhibitor on the peptide-mediated migration of wt-CFBE cells.
688 Before removing the Ibidi culture insert, cells were pre-incubated with 5 µM AG1478 for 30 min
689 and subsequently treated with 10 µM Esc(1-21) or 1 µM Esc(1-21)-1c. Some samples were treated
690 with the peptide alone at the same concentration or with 5 µM AG1478. Cells incubated with
691 MEMg served as a control (Ctrl). Samples were photographed at different time intervals as
692 indicated, and the percentage of cell-covered area was calculated and reported on the y-axis. The
693 data are the mean of four independent experiments ± SEM. The levels of statistical significance
694 between Ctrl and AG1478-treated samples or between samples pre-treated with AG1478 before
695 incubation with the peptide and those treated with the peptide alone at the corresponding time
696 intervals are indicated as follows: *, $p<0.05$; ****, $p< 0.0001$.

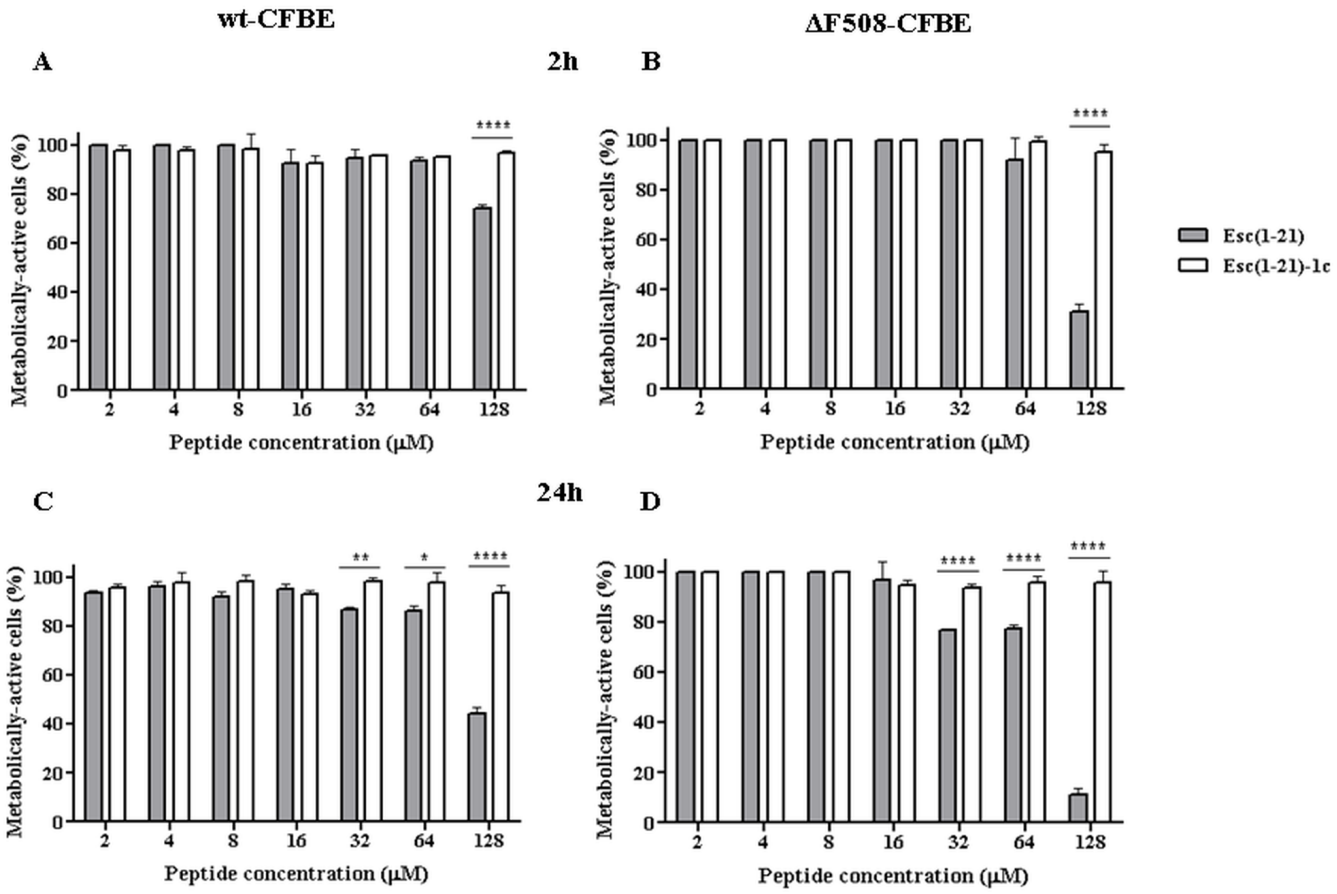
697

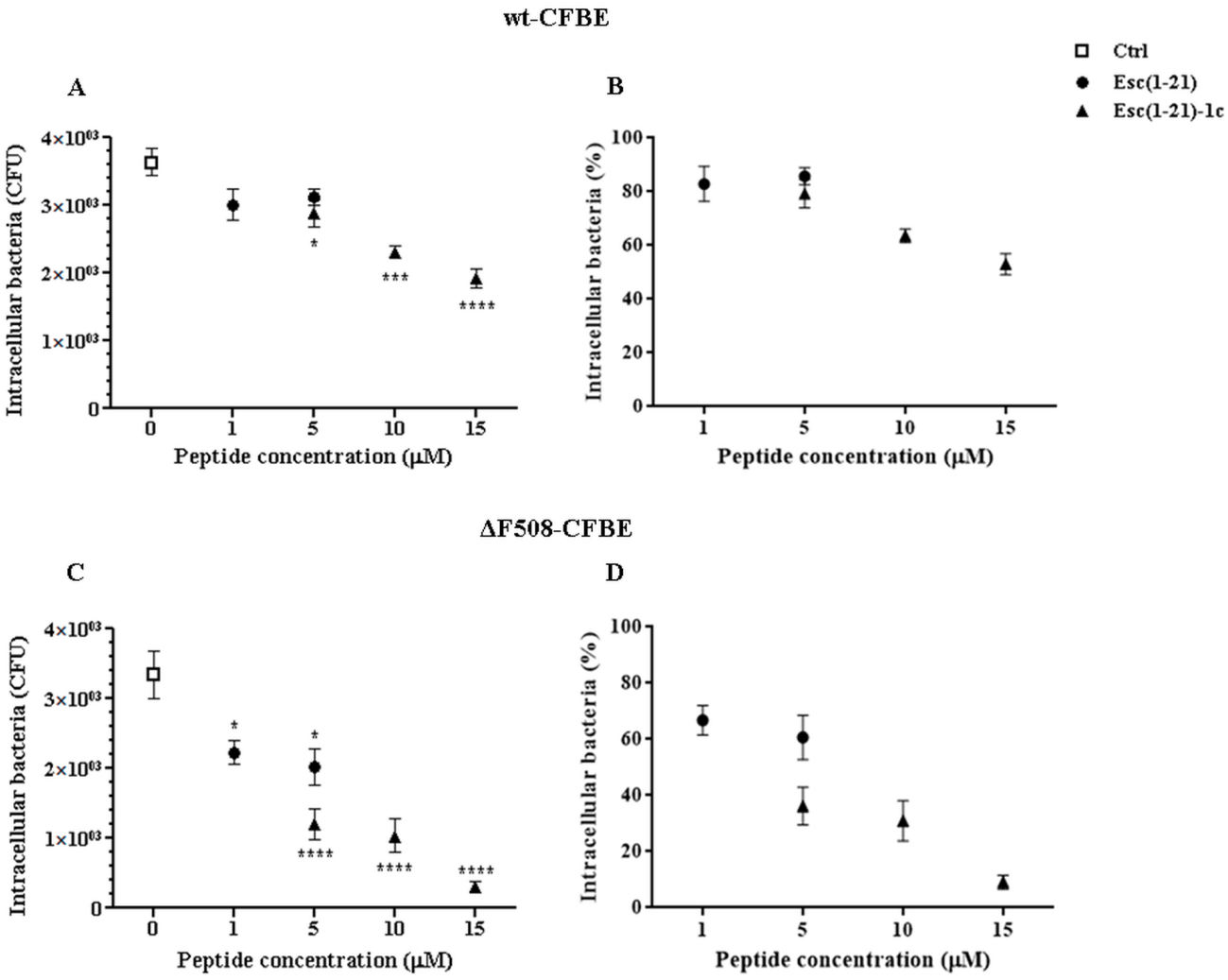
698 **FIG. 6.** Confocal laser-scanning microscopy images of wt-CFBE and ΔF508-CFBE cells treated
699 with rho-Esc(1-21) (A, B) rhodamine alone (C, D) or rho-Esc(1-21)-1c (E, F) at different times.
700 After treatment with the peptide (or rhodamine), cells were stained with DAPI for nuclei detection.
701 DAPI fluorescence, rhodamine-labeled peptide (or rhodamine) signal as well as the overlay of the
702 two fluorescent probes are shown. All images are z section taken from the mid cell height. All bars
703 represent 10 µm.

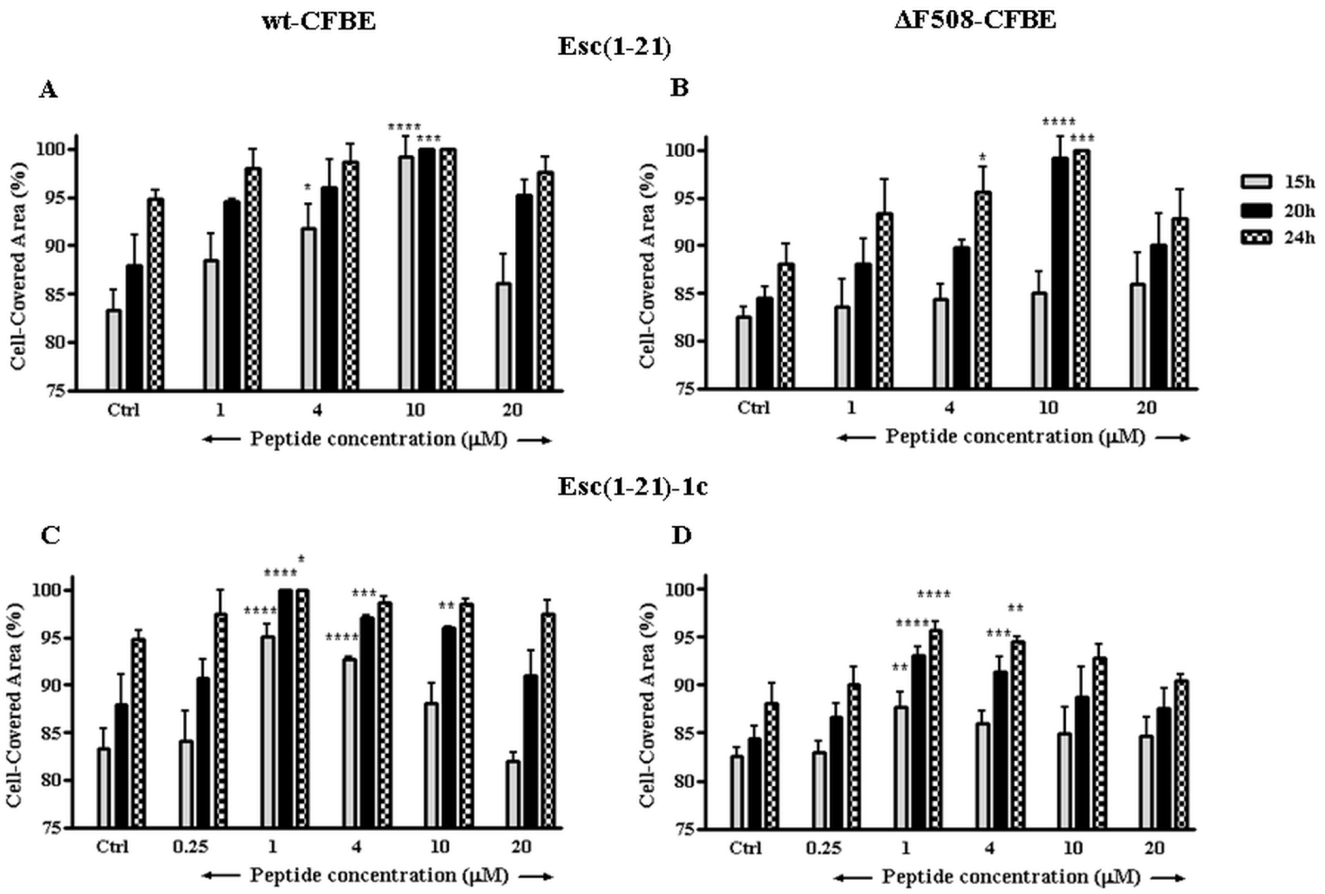
704

705 **FIG. 7.** Peptides distribution between cytoplasm and nucleus in wt-CFBE and ΔF508-CFBE cells.
706 Each peptide was tested on a minimum of 35 cells. The ratio between the fluorescence intensity of
707 rhodamine-labeled peptides in the cytoplasm *versus* the nucleus was calculated for each cell and the
708 mean value was reported on the y-axis ± SEM. If the ratio is equal to 1, this means that the peptide

is evenly distributed all over the cell. When the ratio is higher than 1, the amount of fluorescent peptide is higher in the cytoplasm compared to the nucleus. The level of statistical significance between the calculated ratios of the two peptides at different time points is indicated as follows: ***, $p < 0.001$.







Antimicrobial Agents and Chemotherapy

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