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Esculentin-1a Derived Peptide Diastereomers to Target *Pseudomonas aeruginosa* Lung Infection in Cystic Fibrosis: From Nature to Bench towards Therapeutic Application

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Introduction

The alarming rise of "superbugs" resistant to the available antibiotics poses a serious threat to global human health and has been predicted as the next pandemic. Experts in the field have estimated that if these pathogens are left untreated, they could lead to a significant increase in the number of infection-related deaths, with about 10 million deaths per year, worldwide, by 2050 [1,2]. Among superbugs, there is *Pseudomonas aeruginosa*, a highly virulent opportunistic Gram-negative bacterium that is very difficult to eradicate, especially because of its capability to form biofilm, where bacterial cells are embedded into an extracellular matrix which confers protection from the host immune response and from traditional antibiotics. This microorganism provokes a large variety of infections, including those found in the lungs of cystic fibrosis (CF) patients [3]. The disease of CF is caused by mutations in the gene encoding the CFTR transmembrane channel, which controls the passage of chloride ions [4,5]. The most common mutation consists in the deletion of phenylalanine 508 (F508del-CFTR), which is associated with misfolding and premature degradation of the mutated protein which also has a gating defect. Therefore, a decreased amount of the defective protein reaches the cell membrane. The impaired function of CFTR leads to the formation of sticky and dehydrated mucus lying in the respiratory tract. This favors the entrapment of inhaled microbes, including *P. aeruginosa,* that rapidly colonizes the lung with deterioration of lung tissue and final failure of respiratory functions. Therefore, treatment of *P. aeruginosa* lung infections in CF may benefit from compound(s) endowed with multiple biological properties, such as antimicrobial peptides (AMPs) [6,7]. We demonstrated how derivatives of the frog skin AMP esculentin-1a hold promise for the development of such new therapeutic agents.

Results and Discussion

We previously demonstrated that the frog skin-derived AMP Esc(1-21) has a rapid killing kinetics against both free-living and biofilm forms of *P. aeruginosa*, with a membrane-perturbing mechanism that reduces resistance, in contrast with traditional antibiotics [8]. Furthermore, it was found to promote re-epithelialization in bronchial cell monolayers, and presumably to accelerate the healing of damaged airway epithelium. This property is not displayed by conventional antibiotics [9,10]. However, before bringing AMPs from the bench to the bedside, we need to overcome some relevant challenges, such as cytotoxicity towards host cells, poor biostability, and limited targeted delivery [11]. We found out that by changing the stereochemistry of two selective L amino acids to the corresponding D-enantiomers, the resulting diastereomer Esc(1-21)-1c carrying D-Leu^{14} and D-Ser^{17} (Figure 1) displays the following enhanced properties.
(i) Lower cytotoxic towards mammalian cells, likely due to its decreased alpha-helical content.
(ii) Higher stability in the presence of proteolytic enzymes abundant in the lungs of CF patients, such as elastase from human neutrophils and from *P. aeruginosa* [9].
(iii) Higher efficacy in eradicating *Pseudomonas* biofilm.

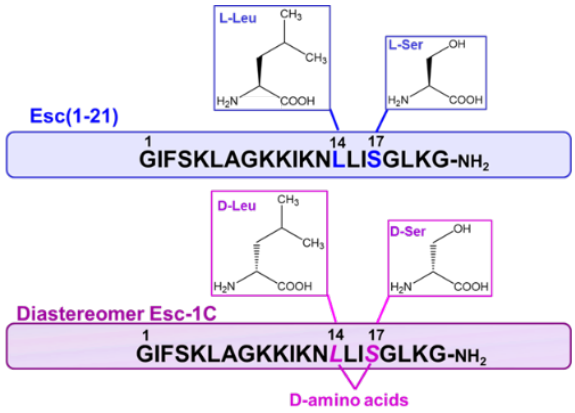

Fig. 1. Primary structure of Esc peptides. The L to D-amino acid changes at positions 14 and 17 are highlighted.

These properties make Esc(1-21)-1c, rather than the corresponding all-L isoform, the more appropriate molecule for *in vivo* studies. By using a mouse model of acute *P. aeruginosa* lung infection, we demonstrated that a single intratracheal instillation of Esc(1-21)-1c, at 0.1 mg/kg, can yield a 2-log reduction in the number of lung bacterial cells 24 h after infection, comparable to the clinically-used lipopeptide colistin [12]. However, colistin quickly generates resistance and, unlike Esc peptides, does not possess any airway wound healing activity, which is relevant to restoring tissue integrity and respiratory function while preventing pathogen penetration.

Considering the role of airway epithelium and CFTR in maintaining lung function and wound repair [13,14], we then evaluated the effect of these peptides on the ion current controlled by the protein channel. Notably, activation of CFTR requires (i) phosphorylation of its R domain by protein kinase A [15] and (ii) binding of ATP to the nucleotides binding domains (NBDs) to elicit their dimerization with the consequent opening of the gate at the level of the transmembrane domains.

Electrophysiological experiments, including measurements of transepithelial conductance across epithelia expressing F508del-CFTR as well as measurements of membrane currents in single cells expressing F508del-CFTR (patch-clamp technique) after correction with lumacaftor (which acts as a protein-folding chaperone to assist the delivery of the mutated protein to the plasma membrane) were performed. Our results demonstrated the ability of these two Esc peptides to restore the activity of F508del-CFTR, likely upon direct interaction with the channel [16]. This effect was more pronounced for the peptide analog carrying a D-phosphoserine at position 17 [16]. Computational studies indicated its interaction with the cytosolic side of the complex made by the two NBDs bound to ATP [16]. The peptide bridges the two NBD domains, thanks to a network of hydrophobic and electrostatic interactions reinforced by hydrogen bonds with Glu^{632} and Arg^{1386}. This is expected to stabilize the heterodimer and presumably the open state of the channel [16].

Based on the antimicrobial activity of Esc peptides, their airway wound healing properties, and CFTR rescue activity, we believe these peptides represent promising candidates for treating lung pathology in CF. However, as mentioned above, before bringing AMPs from the bench to the bedside, we must consider their limited diffusion through biological barriers (such as bronchial mucus) and therefore limited delivery to the target site. To this aim, the peptides were incorporated into biodegradable polymeric nanoparticles (NPs) made of poly(lactide-co-glycolide) (PLGA), coated with polyvinyl alcohol (PVA) to stabilize them [17]. They resulted in having a spherical shape with a hydrodynamic diameter lower than 300 nm, a suitable size for pulmonary administration, and reaching the deepest part of the lungs. Such NPs were found to facilitate the diffusion of the encapsulated peptide through an artificial mucus layer and to prolong the *in vitro* antipseudomonal activity compared to the corresponding free counterpart (Table 1).

Furthermore, NPs were found to significantly potentiate the *in vivo* antibacterial activity of the peptides at the lung level upon delivery in the conductive airways. Overall, these findings have contributed to highlighting the potential of these peptides as new multi-functional drugs for topical treatment of lung pathology, especially in CF patients, and to suggest the engineered PLGA NPs as attractive nanocarriers for delivery of AMPs in the conductive airways and to improve their antimicrobial efficacy compared to the corresponding soluble free counterparts (Figure 2).

Table 1. Antibacterial activity of Esc peptides in the free or encapsulated form, expressed as optical density at 590 nm ±standard deviation (S.D.) after 24 and 72 h treatment. The absorbance values were taken from ref [17].

Treatment	*Absorbance of samples at 590 nm*	
	After 24 h	After72 h
PVA-PLGA NPs*	0.6823 ± 0.075	0.7415 ± 0.065
Esc(1-21)	0.0121 ±0.007	0.5400 ±0.06
Esc(1-21)_PVA-PLGA NPs	0.2785 ±0.031	0.2653 ±0.044
Esc(1-21)-1c	0.0400± 0.008	0.5353 ±0.022
Esc(1-21)-1c_PVA-PLGA NPs	0.3781 ±0.037	0.3448 ±0.05
Untreated control cells	0.6545±0.012	0.7802 ±0.059

**Bare PVA-PLGA NPs were included for comparison as well as untreated control cells*

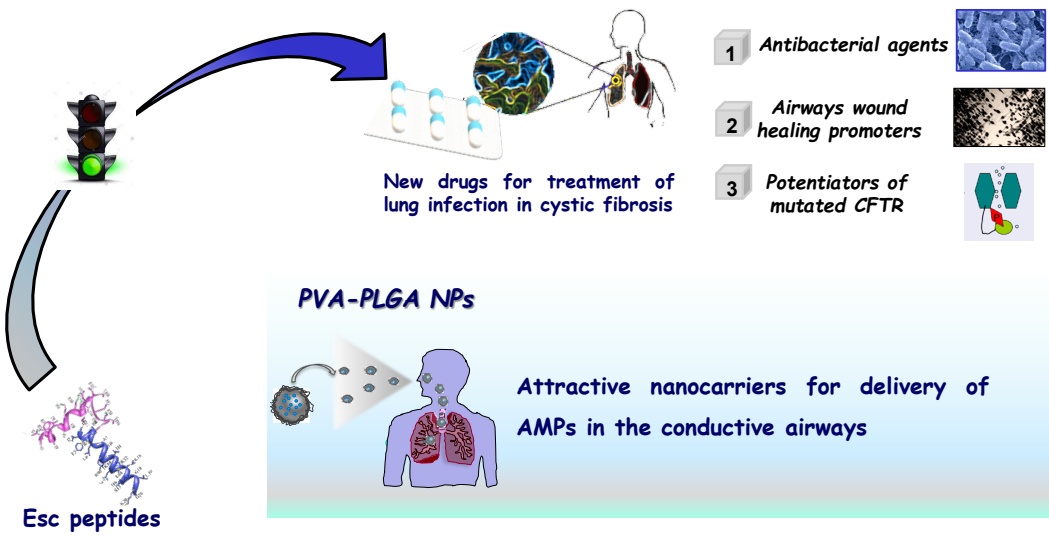

Fig. 2. Schematic representation of the main advantageous properties of Esc peptides and PVA-PLGA NPs for treatment of lung pathology in CF.

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