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Selective toxicity of engineered lentivirus lytic peptides in a CF airway cell model

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Abstract

Lentivirus lytic peptides (LLPs) are derived from HIV-1 and have antibacterial properties. LLP derivatives (eLLPs) were engineered for greater potency against *Staphylococcus aureus* (SA) and *Pseudomonas aeruginosa* (PA). Minimum bactericidal concentration (MBC) was determined in low and physiologic salt concentrations. MBC was decreased against SA and equivalent against PA in physiologic salt when compared to the parent compound LLP1. In a novel cystic fibrosis (CF) airway cell model, one derivative, WLSA5, reduced the number of adherent PA and only moderately affected CF cell viability. Overall, eLLPs are selectively toxic to bacteria and may be useful against CF airway infections.

Keywords: Antimicrobial peptide; Cystic fibrosis; Airway epithelial cells; Selective toxicity

1. Introduction

Host derived antimicrobial peptides play an important role in controlling bacterial infections, such as those associated with cystic fibrosis (CF) [24]. They are secreted by macrophages and epithelial cells and are found in biologically active concentrations on mucosal surfaces, particularly those continually exposed to infectious pathogens [25]. The identification of many structurally diverse host derived peptides suggests that these compounds have evolved to perform optimally in a given environment against commonly encountered microbial pathogens [11]. For example, many host derived antimicrobial peptides are inhibited in the presence of high NaCl concentrations. Antimicrobial peptides, such as human β-defensin-1, are thought to be inactivated in CF airway infection because this peptide functions in the low ionic environment of the normal host's airway surface fluid [9]. Previous work [3] suggests that host defenses can be augmented to control bacterial infection through the delivery of antimicrobial peptides. These peptides can be host derived or synthetic compounds with broad-spectrum antimicrobial activity.

The lentivirus lytic peptides (LLPs) represent a newly described sequence that is similar to the group of cationic, amphipathic peptides derived from an HIV-1 transmembrane protein with antibacterial properties [4,14,20–23]. Among the many antimicrobial peptides currently described in the literature, the α-helical LLPs are structurally similar to the magainins [22] and LL37, or human cathelicidin [2]. In standard broth dilution assays [21], these peptides compared favorably on a molar basis with the potency of magainin-2 or LL37. The LLPs also demonstrated broad antimicrobial activity against gram positive and negative bacteria, including those highly resistant to conventional antibiotics. Based on these findings, we have proposed that

Abbreviations: ASL, airway surface liquid; BCC, *Burkholderia cepacia* complex; CF, cystic fibrosis; cfu, colony forming units; DMEM, Dulbecco's modified Eagle medium; HPC, human placental collagen; LLPs, lentiviral lytic peptides; LLP1, lentiviral lytic peptide-1; MBC, minimum bactericidal concentration; PB, potassium phosphate buffer; R_{te}, transepithelial resistance; TSA, tryptic soy agar

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this class of antimicrobial peptides be studied further in the setting of CF airway infection.

Since reporting the antibacterial activity of LLP1 [20], we have successfully engineered a number of derivatives by optimizing the cationic, amphipathic character of the original LLP1 sequence and increasing peptide length and measured their ability to kill clinical bacterial isolates on a molar basis using a standard in vitro broth dilution assay. Bacterial killing assays do not accurately reflect the conditions of the chronic bacterial bronchitis characteristic of CF with respect to the effects of airway secretions on peptide activity. Because antibiotics such as gentamicin are less potent in CF sputum due to binding of components such as mucins or leukocyte DNA [12], we have developed an airway epithelial cell co-culture model to evaluate the selective toxicity of the engineered LLP derivatives toward bacteria adherent to eukaryotic cells.

The focus of this study was to assess the in vitro activities of LLP1, and three of its engineered derivatives, SA5, LSA5, and WLSA5, against *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Burkholderia cepacia*, three pathogens commonly encountered in CF airway disease. Based on these findings, the engineered variant WLSA5 was chosen as the lead compound. This peptide was shown to be active against clinical isolates of *B. cepacia* and *P. aeruginosa* adherent to CF airway epithelial cells. In this model, WLSA5 demonstrated toxicity to the airway epithelial cell layer similar to that of the host derived antimicrobial peptide LL37, and was reversible over time. Overall, we were able to show that these peptides were selectively toxic to bacterial pathogens, which may make them useful as agents to treat the chronic bacterial airway infection seen in CF.

2. Materials and methods

2.1. Strains and reagents

Bacterial strains used in this study consisted of the laboratory strain of *P. aeruginosa* (strain 1244), a clinical strain of *B. cepacia* (genomovar 2, identified in the laboratory of Dr. J.J. LiPuma, University of Michigan), and a clinical isolate of *S. aureus* identified by the clinical microbiology laboratory at the Children's Hospital of Pittsburgh. Bacterial isolates were maintained on tryptic soy agar (TSA) (Difco, Detroit, MI, USA) or as frozen stock solutions prior to testing.

2.2. Peptide synthesis

The peptides used in this study were synthesized as C-terminal amides using previously described Fmoc synthesis protocols [7,14]. The molecular weights of the peptides used in this study are: LLP1 3308 Da; SA5 3463 Da; LSA5 3817 Da; and WLSA5 4180 Da. Synthetic peptides were characterized and purified by reverse-phase high-performance liquid chromatography, and the identity of each peptide confirmed by mass spectrometry. Peptides were quantified, in micromolar concentrations, using a sensitive ninhydrin assay as previously described [21].

2.3. In vitro bacterial killing assay

These were conducted as previously described [20] using clinical or laboratory isolates of several gram positive and gram negative bacteria. Briefly, bacterial suspensions (0.5×10^6 cfu/ml) were incubated for one hour at 37 °C with LLP1 or its analogs in concentrations ranging from 0.05 to 100 μM in 10 mM potassium phosphate buffer (PB), pH 7.2. To approximate physiologic salt conditions, 150 mM NaCl was added to 10 mM PB where noted. Serial dilutions were performed and each plated on TSA; colonies were counted after 24 h. The minimum bactericidal concentration (MBC), the lowest peptide concentration sufficient to cause 99.9% (3-log killing) when compared to untreated bacterial suspensions, was determined by comparing colony counts on the controls with those on the undiluted test plates. Values were expressed on a molar basis, with a lower MBC corresponding to increased peptide potency. The results were expressed as an average of MBC values obtained from two to four independent experiments. Values are representative of results from three or more separate experiments. MBCs may be converted to micrograms per milliliter by dividing molecular mass (in Daltons) by 1000 and then multiplying by the value in micromolar units.

2.4. Airway epithelial cell culture

Primary cultures of cystic fibrosis airway epithelial cells were prepared using a previously described protocol approved by the Investigational Review Board of the University of Pittsburgh. Bronchial epithelial cells were obtained following protease digestion of airway tissue (second to sixth generation bronchi) dissected from the excess pathological specimens remaining after lung transplantation. After centrifugation and washing, the epithelial cells were plated in hormonally defined media (BEGM, Clonetics Corp., San Diego, CA) into tissue culture flasks coated with type VI human placental collagen (HPC, Sigma, St. Louis, MO). Over 10–14 days, phenotypically undifferentiated cells proliferated to form a monolayer. When cell growth reached 80–90% confluence, they were trypsinized and seeded onto HPC-coated filters [6]. These cells were grown in a 1:1 mixture of Dulbecco's modified Eagle medium (DMEM) and Ham's F12 with 2% Ultroser G (Bioseptra, France), on semi-permeable supports at an air–liquid interface in order to allow differentiation [1]. The cell monolayers were washed with antibiotic-free buffers and placed in antibiotic-free medium 48 h prior to all bacterial adherence and killing assays. After approximately 21 days, cells developed structural features of cellular differentiation that included cilia formation, expression of the transmembrane

mucin MUC1, development of a transepithelial resistance of greater than 350 Ω cm^2 and a short circuit current that was inhibited by amiloride [6].

2.5. Measurement of transepithelial resistance

To assess the toxicity of antimicrobial peptides for differentiated airway epithelial cells, transepithelial resistance (R_{te}) measurements were made on polarized epithelial cell monolayers of airway epithelial cells grown on permeable supports. Serial measurements of R_{te} were made using an EVOM Epithelial Voltohmmeter (World Precision Instruments, Inc., Sarasota, FL), as previously described [15].

Briefly, baseline R_{te} measurements were made on each filter, and then antimicrobial peptide in a minimum volume of DMEM, or DMEM alone as a control, was added to the apical surface of the monolayers. Fifteen minutes later, 100 μl of DMEM was added to the apical surface to allow for R_{te} measurements at 0.25, 0.5, 1, 2, 3, 4, 8, and 18 h after addition of the peptides or media control. The raw R_{te} measurements for each filter were expressed as a percentage of baseline. Results from experiments on separate days were grouped and analyzed for statistical significance using ANOVA.

2.6. Adhesion assays using viable bacterial counts

Using a standard bacterial adherence assay, we investigated the binding of a standard laboratory strain of *P. aeruginosa*, PA 1244. This strain was grown in shaking culture overnight at 37 °C in LB; the culture was allowed to equilibrate at room temperature without shaking for one hour to allow for re-piliation and was then diluted further to yield a concentration of approximately 1×10^6 cfu/ml. A 100 μl aliquot was added to human CF airway epithelial cells grown on filters to a density of 1×10^5 cells per filter. The bacteria and HBE cells were allowed to incubate for 1 h at 37 °C. After thorough washing to remove excess non-adherent bacteria from each filter, trypsin/EDTA was added to each filter and incubated for 15–20 min at 37 °C to release adherent bacteria from the epithelial cells. After serially diluting the trypsinized filters and plating the contents onto TSA, the number of colony forming units (cfu) of bacteria adherent to each cell type was quantified after incubation at 37 °C for 24 h.

2.7. Scanning electron microscopy of cells on filters

Bacteria were added to CF airway epithelial cell filters at concentrations of 1×10^7 and 1×10^8 cfu/ml, and incubated for 1 h at 37 °C, followed by serial washes with sterile buffer. Cells on filters were fixed overnight in 2.5% glutaraldehyde in PBS at 4 °C. Samples were washed three times in PBS, post-fixed for 1 h in aqueous 1% osmium tetroxide, then washed three times in PBS. Samples were dehydrated through a graded ethanol series (30–100%), further dehydrated by three additional 15 min washes with absolute ethanol, then critical point dried (Emscope CPD 750, Ashford, Kent, UK). Samples were removed from inserts and mounted onto aluminum stubs then sputter coated with gold/platinum (Hummer VI, Technics West, San Jose, CA). Samples were viewed in a JEOL JSM-T300 scanning electron microscope (Peabody, MA) at 20 kV.

2.8. Susceptibility of adherent P. aeruginosa to antimicrobial peptides

Modifying the adherence assay described above in two ways allowed for testing the efficacy of treatment with antibacterial peptides. The first modification involved propagating the airway epithelial cells in antibiotic-free media for two days prior to performing the experiment. The second modification involved exposure of the adherent bacteria (after the wash step described above) to increasing concentrations of antimicrobial peptide and then determination of the minimum bactericidal concentration (MBC) killing 99.9% of the total number of bacteria adherent to the epithelial cells. The results shown were representative of three experiments as described for the standard bacterial broth dilution assay.

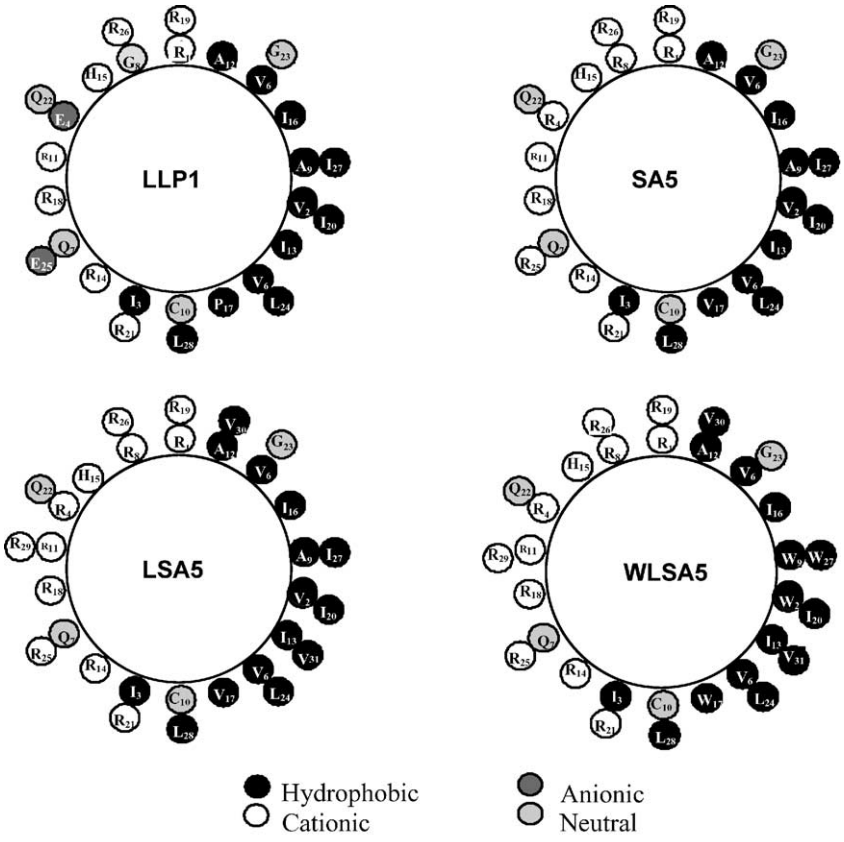
3. Results

3.1. Engineering highly active LLP1 derivatives

Previous studies of LLP1 demonstrated our ability to modulate potency and selective toxicity using single amino acid changes to the primary 28-residue sequence [21]. For example, alteration of a single Arg residue predicted to exist on the hydrophilic face of the α-helix to Glu (designated Analogue3) completely abrogates the bactericidal activity of this peptide. Conversely, changing the single Glu in the native LLP1 sequence to Arg (designated Analogue5) nearly increases the MBC by an order of magnitude. Based on this experience, LLP1 derivatives were engineered further by optimizing cationic amphipathic α-helical structure, increasing length, and substituting tryptophan and arginine residues on the hydrophobic and hydrophilic faces. The primary sequences and helical wheel representations of the peptides used in this study are shown (Fig. 1). SA5 substitutes three Arg residues for one Gly and two Glu that model on the hydrophilic face of the LLP1 sequence and one Val for one Pro that models on the hydrophobic face. LSA5 extends the length of the SA5-modeled α-helix by one turn, preserving its amphipathic, Arg-rich cationic character. WLSA5 was designed as a peptide with four Trp on the hydrophobic face, since Trp residues have been shown to intercalate optimally into bacterial membranes [8,17].

3.2. Antimicrobial activity of LLP derivatives

We tested the engineered LLP derivatives in low and physiologic salt concentrations (10 mM PB ± 150 mM



LLP1: RVIEV VQGAC RAIRH IPRRI RQGLE RIL

SA5: RVIRV VQRAC RAIRH IVRRI RQGLR RIL

LSA5: RVIRV VQRAC RAIRH IVRRI RQGLR RILRV V

WLSA5: RWIRV VQRWC RAIRH IWRRI RQGLR RWLRV V

Fig. 1. Helical wheel depiction (upper panel) and primary sequences of the engineered peptides used in this study. Black circles represent hydrophobic residues; white circles represent cationic residues; light grey circles represent neutral residues; and dark grey circles represent anionic residues.

NaCl) using a previously described standard broth dilution assay [20] and determined their MBCs against *S. aureus* and *P. aeruginosa*, two pathogens typically seen in CF airway disease. This method varies slightly from the standard NCLSS-approved assay by measuring the viability of organisms following a peptide exposure time of 1 h instead of 24 h. Under these conditions we have shown cationic antimicrobial peptide activity to occur within minutes of exposure [20]. In the presence of low salt concentrations, the engineered LLP derivatives SA5, LSA5, and WLSA5 all showed equivalent activity against *S. aureus* when compared to the parent LLP1 (MBC 0.8–1 μM). However, in physiologic salt concentrations, the engineered LLP derivatives SA5, LSA5, and WLSA5 all retained their activity (MBC 0.5, 1, and 1 μM, respectively) when compared to LLP1 (16 μM). Against *P. aeruginosa*, the activity of all four peptides was equivalent (MBC 0.8–1 μM) and did not increase in the presence of low and physiologic salt concentrations (Table 1). The antimicrobial activity of these peptides have been demonstrated to be the result of compromise of the individual bacterial membrane [16] and not the result of peptide-induced aggregation.

Experience in our laboratory has demonstrated that LSA5 and WLSA5 are active against multiple bacterial strains, including mucoid *P. aeruginosa*, methicillin-resistant *S. aureus*, *N. gonorrhoeae*, *C. trachomatis*, among others (data not shown). Furthermore, previous studies investigating cationic antimicrobial peptides have not demonstrated significant antimicrobial activity against *Burkholderia cepacia* complex (BCC) [10,18]. When the LLP derivatives were tested against a clinical isolate of BCC (genomovar 2), we found that WLSA5 displayed antimicrobial activity in the 1–3 μM range, while LSA5 was not active (Fig. 2). Based on this result, WLSA5 was studied further for its

Table 1
MBC of eLLPs against CF pathogens in the presence of NaCl

Peptide	*Staphylococcus aureus*		*Pseudomonas aeruginosa*	
	10 mM PB	10 mM PB + 150 mM NaCl	10 mM PB	10 mM PB + 150 mM NaCl
LLP-1	0.8	16	1	1
SA-5	1	0.5	1	1
LSA-5	1	1	0.8	0.8
WLSA-5	1	1	1	1

Minimum bactericidal concentrations (MBCs) of the engineered LLP derivatives against *S. aureus* and *P. aeruginosa* in the presence of low (10 mM PB) and physiologic (10 mM PB + 150 mM NaCl) salt concentrations. Values are expressed in micromolar concentrations and are representative of standard broth dilution assays performed in triplicate. The engineered LLP derivatives SA5, LSA5, and WLSA5 all show equivalent activity against *S. aureus* in low salt concentrations and preserve their activity in physiologic salt concentrations when compared to LLP1. Against *P. aeruginosa*, the activity of all four peptides was equivalent and did not increase in the presence of low and physiologic salt concentrations.

activity against *P. aeruginosa* adherent to airway epithelial cells.

3.3. Peptide activity in the airway epithelial cell culture model

An assay for selective toxicity was designed in which bacteria bound to cultured human airway epithelial cells were exposed to varying concentrations of antimicrobial peptide. Killing of bacteria adherent to an intact airway epithelial cell monolayer was monitored using viable counts while toxicity to these host cells was measured using transepithelial resistance. To confirm bacterial adhesion and determine distribution, scanning electron micrographs of CF airway cell filters showed adherence of *P. aeruginosa* to differentiated and undifferentiated epithelial cells as well as association with the overlying mucus blanket (Fig. 3). The majority of bacteria adhered to mucus, as reported recently [27].

As shown in Fig. 4, WLSA5 was more active than LLP1 against *P. aeruginosa* adherent to CF airway epithelial cells. Under the conditions of the airway epithelial cell culture assay described above, WLSA5 reduced the number of surviving bacterial colonies by greater than two orders of magnitude at both 50 and 100 μM. WLSA5 killed *P. aeruginosa* adherent to CF airway epithelial cells at a higher concentration than that used against the same bacterial cell number

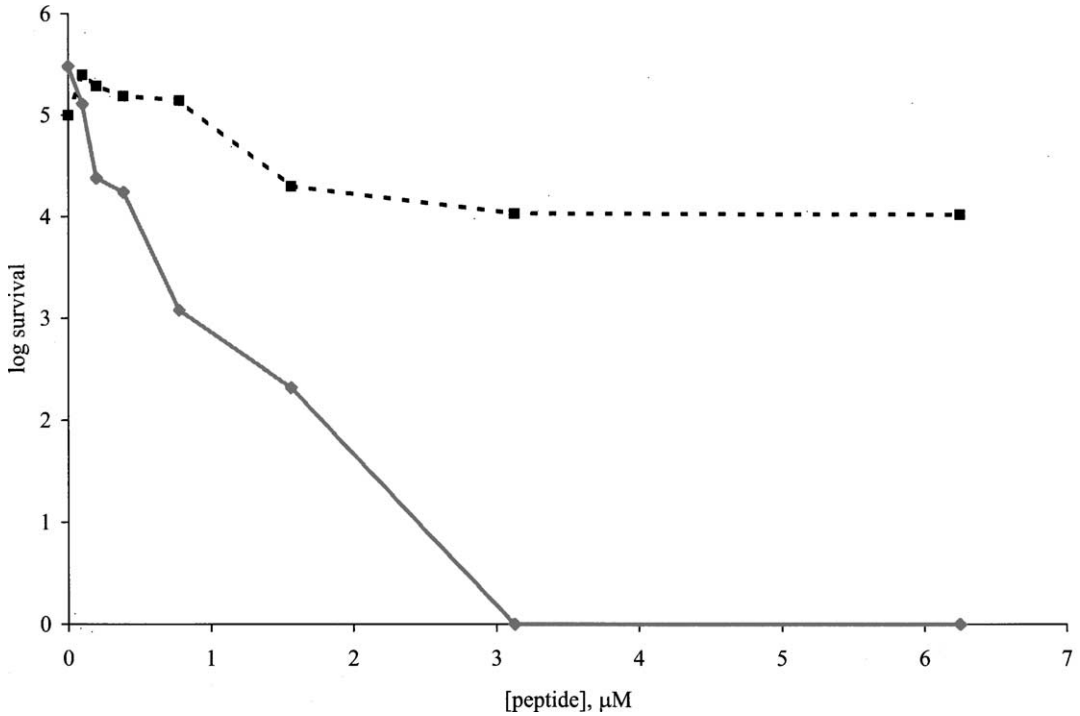


Fig. 2. Concentration-dependent killing of BCC (genomovar 2) by the engineered LLP derivatives. A reduction in surviving bacterial colonies by greater than three orders of magnitude is demonstrated by WLSA5 (solid line) but not LSA5 (dashed line). These data indicate that inclusion of tryptophan residues in an engineered antimicrobial peptide formulation may dramatically increase the spectrum of activity.

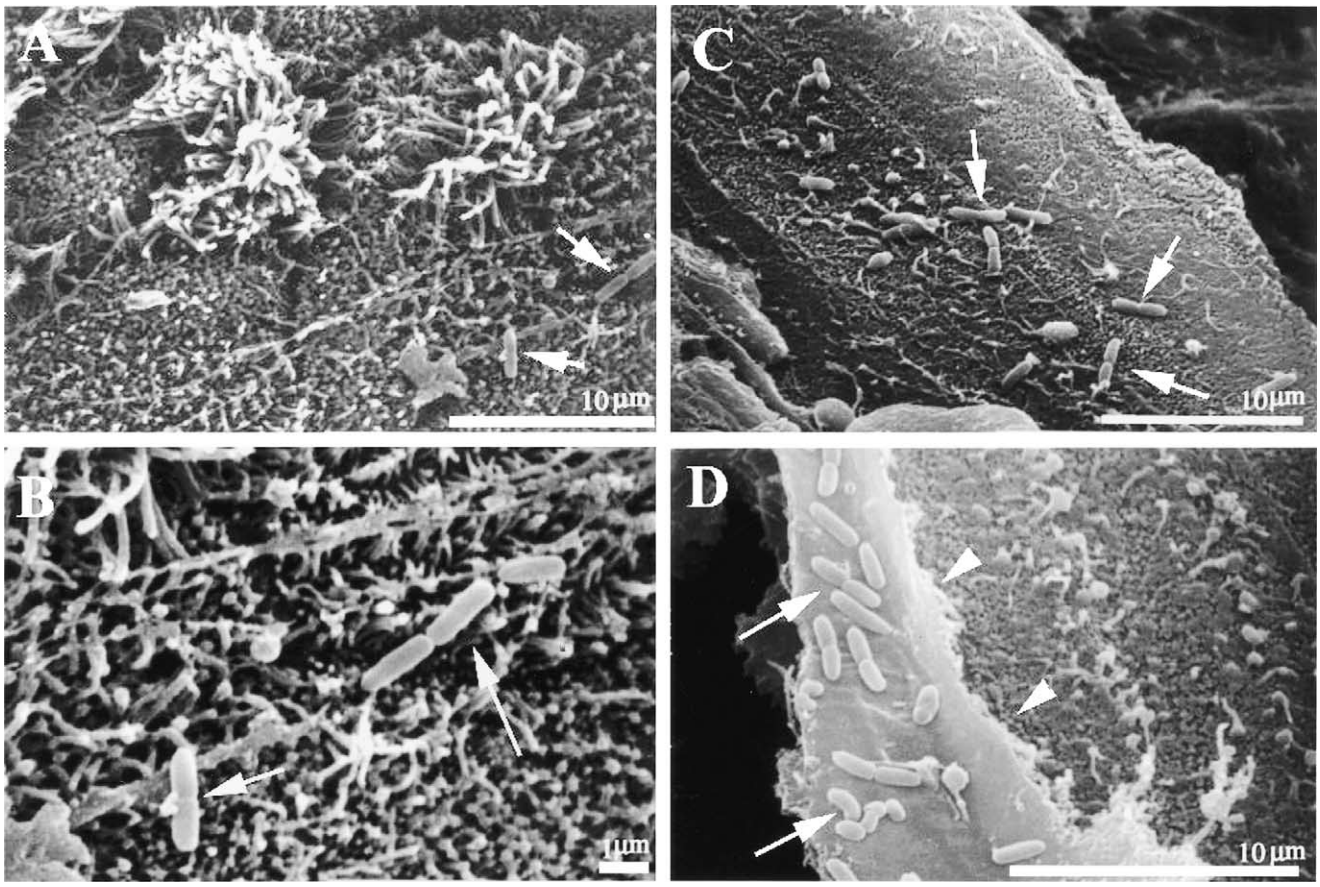


Fig. 3. Scanning electron micrographs of primary CF airway epithelial cells with adherent *P. aeruginosa*. Panels A and B (a tenfold magnification) show *P. aeruginosa* (arrows) attaching to microvilli but not to the cilia of the differentiated primary cell monolayer. Panel C demonstrates that bacteria attach to the surface of an epithelial cell while Panel D shows a greater number of bacteria adherent to the overlying mucus blanket (arrowheads).

in broth culture. These data suggest that when antimicrobial peptides were tested in an environment more complex than broth culture, as in the context of *P. aeruginosa* adherent to epithelial cells, antimicrobial activity was reduced but not negated.

3.4. Toxicity of antimicrobial peptides to human airway cells

Transepithelial resistance measurements were made on polarized monolayers of primary human airway epithelial cells to assess the toxicity of WLSA5. The toxicity of LL37, an antimicrobial peptide secreted in human airway [2], was examined in parallel to determine relative toxicity. Addition of 5 or 10 μM of WLSA5 to the apical compartment of airway cells grown at an air–liquid interface did not significantly alter R_{te} at 0.5, 1, 2, 3, 4, 5, 6, 24, or 48 h. At 10 μM LL37, decreases in R_{te} were statistically different from control at 1, 2, 3, and 4 h, but not at other time points (data not shown). At 50 μM, the difference from control approached but did not meet the criteria for statistical significance ($P = 0.055$ at 0.5 h and 0.058 at 4 h). Addition of LL37 at 50 μM caused a significant reduction in R_{te} at 0.5, 1, 2, 3, 4, 5, 6, and 24 h, with a return to baseline by 48 h. R_{te} returned to baseline 48 h after exposure to both peptides at a concentration of 50 μM (Fig. 5). Thus, both LL37 and WLSA5 reversibly altered the transepithelial resistance of airway cell monolayers, and the effects of the engineered peptide WLSA5 were no greater than those seen with LL37.

3.5. Selective toxicity of WLSA5 for bacteria bound to the primary epithelial cells used in this assay

Because WLSA5 was the best candidate peptide that emerged from this study, a co-culture experiment was designed to investigate whether WLSA5 was selectively toxic for the *P. aeruginosa* bound to these cells. Fig. 6 shows the relationship of peptide concentration to bacterial killing and the percent change in the transepithelial resistance of the epithelial cell monolayer. The bacterial killing assay was adapted to CF airway epithelial cells and *P. aeruginosa* in co-culture. Toxicity following peptide exposure to the epithelial cells was measured as a percent change in the transepithelial resistance of the intact cell monolayer. These

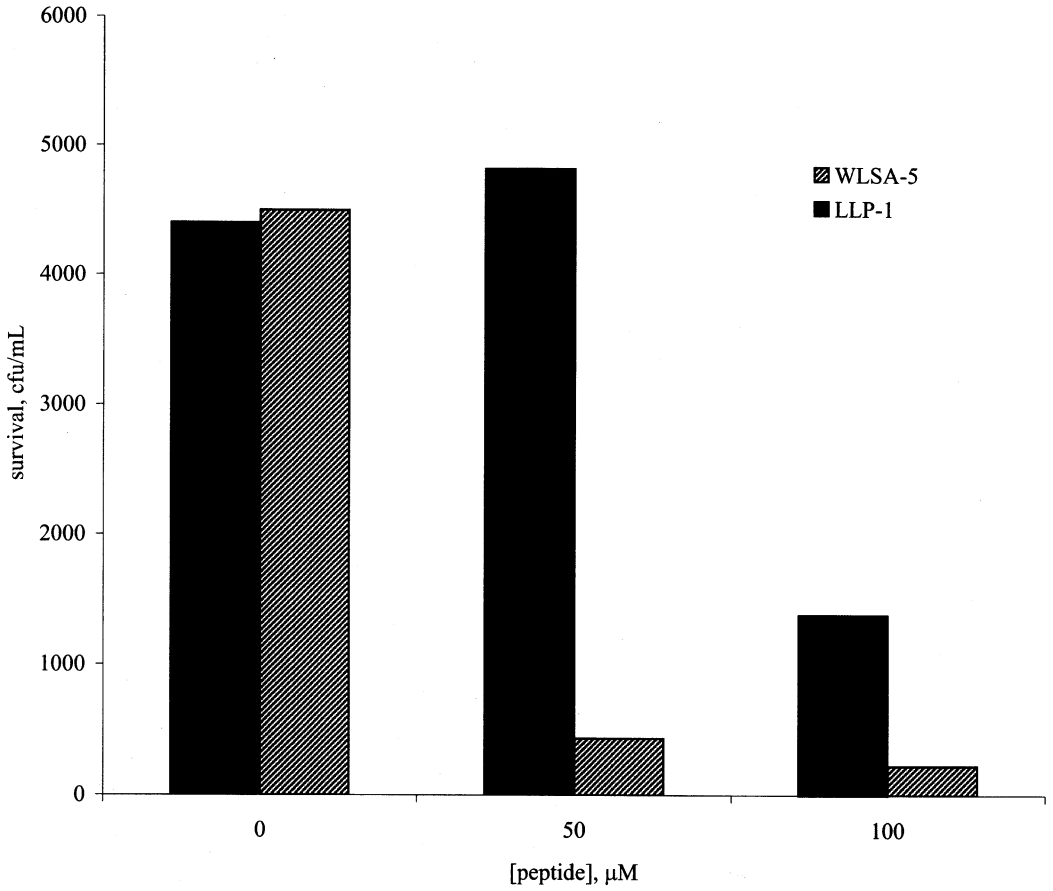


Fig. 4. Killing of *P. aeruginosa* bound to human bronchial epithelial cells in primary culture. In this representative experiment peptides were added at 0, 50 and 100 μM. Note the profound effect that tryptophan residues have upon the activity of these peptides against bacteria in cell culture.

results indicated that a reduction in bacterial load by three orders of magnitude was achieved at peptide concentrations that only moderately affected the cell monolayer. Taken together with the data from Fig. 5, the toxicity of the peptide to the epithelial cells was both transient and recoverable with efficient bactericidal activity.

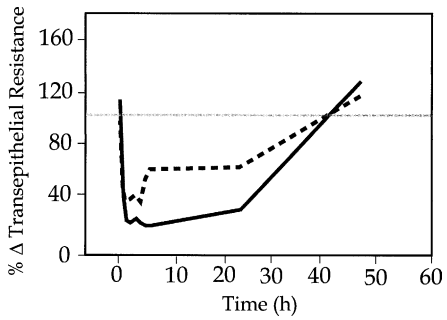


Fig. 5. Toxicity of WLSA5 (solid line) for human bronchial epithelial cells. Serial R_{te} measurements were performed on polarized monolayers of primary human airway epithelial cells and compared to the human antimicrobial peptide LL37 (dashed line). On a molar basis, both peptides demonstrate toxicity (as defined by a reduction in transepithelial resistance, R_{te}) from which the cells recover over time.

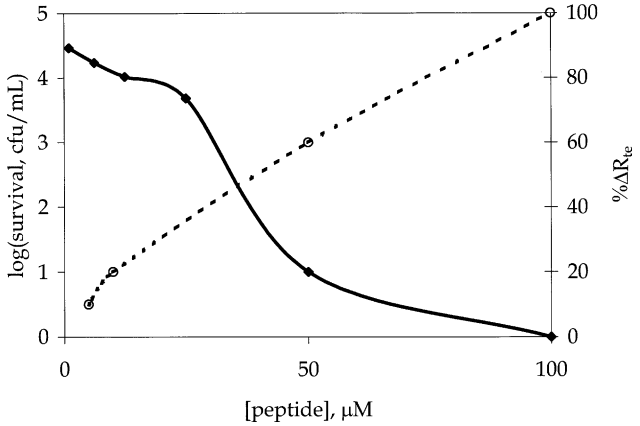


Fig. 6. Selective toxicity of WLSA5 for *P. aeruginosa* over human bronchial epithelial cells in primary culture as a function of peptide concentration added. The cells with adherent bacteria were exposed to peptide for 15 min; shown are the mean R_{te} (as a percentage of control) of triplicate filters from one representative experiment that was repeated three times. On the left axis (solid line) is the ability to decrease the concentration of bacteria associated with the cells in culture. On the right axis (dashed line) is the maximum change in R_{te} associated with WLSA5 addition to the bacterial/eukaryotic co-culture system.

4. Discussion

In contrast to host-derived antimicrobial peptides, which have evolved for the purpose of bacterial killing on mucosal surfaces, the LLPs represent a class of compounds derived from discrete segments of the cytoplasmic tail of the lentiviral transmembrane protein [4,5,13,14,21–23,28]. The LLPs contribute to HIV-1 pathogenesis by altering membrane permeability and sequestering calmodulin [4,22,23,28] and have also been proposed to play a role in natural endogenous reverse transcriptase activity [29]. These processes have been hypothesized to contribute to HIV-1 infection through the inhibition of T-cell activation and dampening of the host inflammatory response [4,14,22,23,28]. More recently, these sequences have been shown to demonstrate potent antimicrobial activity [21].

When modeled as an amphipathic α-helix, LLP1 exhibits structural and functional characteristics similar to host derived antimicrobial peptides. We demonstrated that an LLP derivative, bis-LLP1, disrupted both the outer and cytoplasmic membranes of a clinically relevant strain of *Serratia marcescens* while the membrane-active antibiotic polymyxin B acted only at the outer membrane even though both had similar antimicrobial potency [16]. LLP1 appears to act by perturbing negatively charged bacterial membranes, and to a lesser extent, mammalian cell membranes and does so differently from the well-characterized antibiotic polymyxin B. The predilection of this peptide for bacterial cells over mammalian cell membranes forms the basis for its selective toxicity.

Subtle changes in the amino acid sequence of LLP1 can profoundly affect its antimicrobial activity, indicating that the LLPs can be engineered for increased potency in a variety of biochemical and infectious conditions [21,23]. In this study, we showed that the engineered derivatives of LLP1 remained active in low and physiologic salt concentrations, as measured by standard MBC assays. Single amino acid additions and substitutions resulted in dramatic changes in activity against clinical isolates of resistant bacterial CF pathogens, as in the case of WLSA5 against *B. cepacia*. This peptide was shown to have selective antimicrobial activity using a novel primary human CF airway epithelial cell culture model with minimal host cell toxicity.

The bacterial/epithelial cell culture model provides information regarding the effects on human epithelial cells but provides little insight regarding in vivo toxicity. It can monitor the toxicity of the engineered peptides based on perturbations in the transepithelial resistance of the intact cell monolayer after treatment with peptide. This model has the advantage of being modified to optimize the conditions under which the peptide is delivered (e.g. pH, ionic strength, or in the presence of inhibitory substances such as mucin or DNA). The LLPs can thus be structurally modified to broaden their spectrum of activity and their salt-sensitivity and be tested in appropriate in vitro and in vivo models. For example, alterations in CFTR may affect host defenses through alterations in the salt and water content of airway secretions [26]. Indirect evidence for this has been provided by Smith et al. [19], who demonstrated diminished bacterial killing in the high salt milieu of the airway surface liquid (ASL). The activity of host derived antimicrobial peptides such as the β-defensins is reduced in high-salt conditions when tested in vitro. Monitoring engineered antimicrobial peptides, such as the LLPs with a relevant cell culture model is an important step toward identifying lead compounds that may lead to finding an effective antimicrobial agent for the management of CF lung disease.

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