

Resistance of the antibacterial agent ceragenin CSA-13 to inactivation by DNA or F-actin and its activity in cystic fibrosis sputum

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Objectives: The goal of this study was to evaluate the effects of DNA and F-actin [polyanions present in high concentration in cystic fibrosis (CF) airway fluid] on the antibacterial activities of the cationic steroid antibiotic CSA-13 and the cationic peptides LL37, WLBU2 and HB71.

Methods: Light scattering intensity was used to evaluate the aggregation of DNA and F-actin by the cationic antibacterial agents. Bacterial killing assays, atomic force microscopy, determination of MIC values and bacterial load of CF sputa were used to determine the bactericidal activity. Inhibition of nuclear factor- κ B (NF- κ B) translocation in human aorta endothelial cells (HAECs) was quantified as an assay of anti-inflammatory action.

Results: CSA-13 is significantly more effective than cationic antibacterial peptides against kanamycin-resistant *Pseudomonas aeruginosa* and less susceptible to inactivation by DNA or F-actin. The concentration of CSA-13 sufficient to decrease the CF sputa bacteria load by \sim 90% is at least 10 times lower than that at which CSA-13 formed aggregates with DNA or F-actin. Both CSA-13 and LL37 prevent lipopolysaccharide-induced translocation of NF- κ B in HAEC, thereby suggesting that these antibacterial molecules might prevent systemic inflammation caused by bacterial wall components.

Conclusions: Charge-based interactions that strongly inhibit the antibacterial activity of host cationic antibacterial peptides present in CF sputa have significantly less effect on molecules from the ceragenin family such as CSA-13 due in part to their smaller net charge and distribution of this charge over a hydrophobic scaffold. CSA molecules therefore have potential for the treatment of chronic infections and inflammation that occur in CF airways and other settings in which extracellular polyanions accumulate.

Keywords: cathelicidin, lipopolysaccharide, nuclear factor- κ B

Introduction

Chronic bacterial infection and airway inflammation are major factors in the pathogenesis of cystic fibrosis (CF) lung disease. Although there appears to be no immune deficiency in patients with CF, the defect in CF transmembrane conductance regulator appears to increase the susceptibility of the lungs to endobronchial infections.^{1–3} Antibacterial properties of CF airway surface fluid were found to be reduced,^{4,5} partially as a result of high ionic strength⁶ and the direct interaction of cationic antimicrobial

peptides with DNA, F-actin⁷ and mucins.⁸ DNA and F-actin, derived mainly from neutrophils, are dispersed throughout CF sputum as large elongated aggregates at concentrations in the range of 1–20 and 0.1–5 mg/mL, respectively.^{9,10} Cationic antibacterial peptides alone are sufficient to aggregate F-actin and DNA because a significant concentration of these otherwise soluble cations is sequestered near the surface of polyanionic DNA or F-actin. Counterion condensation overcomes the strong electrostatic repulsions between the filaments, resulting in the lateral association of such filaments into bundles and other

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aggregates that can be redissolved by the addition of co-ions or by the digestion of the filaments.^{7,11} The presence of DNA and F-actin in CF sputum may also promote the ability of the most common CF lung pathogen *Pseudomonas aeruginosa*³ to produce a high level of alginate, to grow as a biofilm in CF lung and to express lipopolysaccharide (LPS) molecules with altered lipid A structure.^{12,13}

Generally, the malfunction of host antibacterial peptides in CF airways is caused by environmental factors present in CF and changes in pathogenic organisms that grow in such an environment. These factors may explain why the addition of exogenous cationic antibacterial peptides to CF sputum has a limited effect on its bacterial load¹⁴ and indicate the need for the generation of new antibiotics that resist the inactivating factors in CF sputum and can kill bacteria in this setting in order to effectively treat CF airway infection. A promising therapeutic approach would be to apply to the lung of CF subjects an inhaled antimicrobial agent with combined antimicrobial and anti-inflammatory activities.^{15,16} Cathelicidin peptides that inhibit multiple antibiotic-resistant pathogens from CF patients,¹⁷ the WLBU2 peptide engineered against *P. aeruginosa* for systemic application¹⁸ and peptides such as HBCM2, HBCM3, HBCP α -2 and HB71 that were selected from 150 antibacterial agents based on a screen against multidrug-resistant clinical isolates of *P. aeruginosa*,¹⁶ represent potential candidates for CF lung infection treatment. However, natural cationic antibacterial peptides such as cathelicidin LL37 have limited antibacterial activity at sites of infection,¹⁹ indicating that *in vivo* their immunomodulatory activity^{20–22} may be as important as their direct bactericidal action. In CF and other settings, exogenous antibacterial factors need to be delivered through fluids containing F-actin and DNA before reaching their bacterial targets, and strategies aimed at dissolving these anionic polymers have been considered.^{7,9}

Membrane-active cationic steroid antibiotics (CSAs) called ceragenins were developed as non-peptide mimics of endogenous cationic antimicrobial peptides. They have antibacterial activity against Gram-positive and Gram-negative bacteria.²³ Similar to positively charged antibacterial peptides,^{24–27} CSA molecules display cationic facial amphiphilicity with charges arranged on one side and hydrophobic residues on the other.²⁸ Because CSA molecules target membranes based in part on electrostatic interactions, they effectively kill different types of bacteria²³ and are unlikely to induce resistance.²⁸ The bactericidal activity of CSA against tobramycin-resistant *P. aeruginosa* isolated from CF patients is the motivation for our study,²⁹ with the goal to evaluate the ability of CSA to kill bacteria in CF sputum and in the presence of polyanionic CF sputum components such as DNA,⁹ F-actin¹⁰ and LPS.³⁰ This study shows that CSA-13 is much more resistant to inactivation by linear polyelectrolytes such as actin or DNA when compared with cationic antibacterial peptides.

Materials and methods

Materials

Tryptic soy broth, Mueller–Hinton (MH) broth and *Pseudomonas* isolation agar were purchased from DIFCO (Sparks, MD, USA). LPS (*Escherichia coli*, serotype O26:B6), lipoteichoic acid (LTA)

(*Staphylococcus aureus*) synthetic melittin, polylysine and human placental DNA were purchased from Sigma (St Louis, MO, USA). Pulmozyme [recombinant human deoxyribonuclease I (rhDNase I)] was from Genentech Inc. (San Francisco, CA, USA). *E. coli* total lipid extract was obtained from Avanti Polar Lipids Inc. (Alabaster, AL, USA). LL37 and WLBU2 peptides were purchased from Bachem (King of Prussia, PA, USA), HB71 peptide was from GenScript Corporation (Piscataway, NJ, USA) and CSA-13 was prepared as described previously.²⁸ See Figure 1 for structures of LL37, WLBU2 and HB71 peptides and ceragenin CSA-13.

Measurement of F-actin and DNA bundle formation

The formation of DNA and F-actin aggregates was detected by monitoring changes in light scattering intensity.⁷ Briefly, 600 μ L samples of 0.1 mg/mL F-actin or 0.3 mg/mL DNA from human placenta in 150 mM NaCl, 5 mM HEPES, 0.2 mM CaCl₂, 0.12 mM MgCl₂, pH 7.35 buffer were placed in high-UV transparent plastic cuvettes and then incubated with different concentrations of LL37 (0.476–95.2 mg/L), WLBU2 (0.34–68 mg/L), HB71 (0.205–307.5 mg/L) or CSA-13 (0.082–820 mg/L) and scattering intensity of 365 nm light was measured at 90° with a Perkin-Elmer LS-5B luminescence spectrometer. HEPES-buffered saline solutions were used for scattering assays in order to compare data with previous reports in which studies of the effects of Ca²⁺ precluded the use of PBS. As evaluation of antibacterial activities is generally performed in PBS or mixtures of PBS and media, we also repeated some scattering assays in PBS to directly compare the effects of antibacterial agents on actin or DNA aggregation with effects of actin and DNA on bactericidal activity.

Antimicrobial activity

The bactericidal activities of LL37, WLBU2, HB71 peptides and CSA-13 were measured as described previously.³¹ Kanamycin-resistant *P. aeruginosa* (PAO1) were grown to mid-log phase at 37°C (controlled by evaluation of optical density at 600 nm) and resuspended in different buffers (PBS, PBS containing 10% deactivated human blood serum, PBS with 5 mM glucose, or HEPES buffer: 150 mM NaCl, 5 mM HEPES with 2 mM MgCl₂,

LL37:

LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLPRTES

WLBU2:

RRWVRRVRRVRRVRRVRRVRRVRR

HB71:

FAKKLAKKLLKLLAKKLLAK

Ceragenin CSA-13:

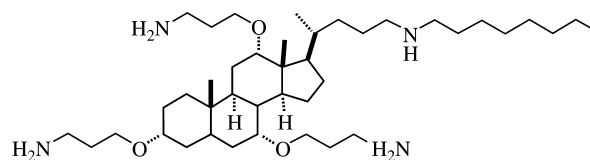


Figure 1. Structure of LL37, WLBU2 and HB71 peptides and ceragenin CSA-13. For amino acids, the one-letter code is used.

Activity of CSA-13 in CF sputum

pH 7.35). The bacteria suspensions were then diluted 10 times in 100 μ L of solutions containing antibacterial agents by themselves or with DNA (0.1–1000 mg/L), F-actin (0.1–1000 mg/L), LPS, LTA or bacterial lipids (BLs) (0.1–10 μ M each). After a 1 h incubation at 37°C, the suspensions were placed on ice and diluted 10–1000-fold. Aliquots (10 μ L) of each dilution were spotted on *Pseudomonas* isolation agar plates for overnight culture at 37°C. The number of colonies at each dilution was counted the following morning. The cfu/mL values of the individual samples were determined from the dilution factor.

Evaluation of MICs

The MICs of antibacterial agents were determined by a microbroth dilution method with MH broth³² supplemented with 2 mM MgCl₂ with and without addition of DNA or F-actin. Bacteria were grown to mid-log phase at 37°C (an optical density of 0.35 at 620 nm) and used at a final concentration of 10⁶ cfu/mL (10 μ L of bacteria inoculated into 10 mL of MH broth). A series of 2-fold dilutions of LL37, WLBU2, HB71 or CSA-13 were prepared from a stock solution and placed in 96-well plates. Dilutions of PAO1 bacteria (when required with DNA or F-actin) were then added. After incubation for 18 h at 37°C, the bacterial concentration was measured as optical density at 595 nm, and the MIC was read as the lowest concentration resulting in the inhibition of detectable bacterial growth.

Cell culture

Human aorta endothelial cells (HAECs) were grown in an incubator at 37°C and 5% CO₂ in EBM-2 with supplements (Cambrex Bio Science, Walkersville, MD, USA). Nuclear factor- κ B (NF- κ B) translocation was measured after a 2 h incubation in serum-free or 2% serum media, after treatment with 10 ng/mL TNF- α (positive control), 0.1 mg/L LPS, or LPS that had been pre-incubated with 1–10 μ M LL37 or CSA-13. The intracellular location of NF- κ B was observed using a monoclonal antibody to the NF- κ B/subunit p65 (Molecular Probes) and cell nuclei were detected by counterstaining with 4',6-diamidino-2-phenylindole dihydrochloride (Sigma). Individual cells were counted to assess NF- κ B localization as nuclear if the two stains co-localized or as cytoplasmic if they did not.³³

Atomic force microscopy

Atomic force microscopy (AFM) imaging of PAO1 bacteria was performed in both hydrated and dry environments on a Multimode™ AFM with a Nanoscope IIIa controller (Veeco, Santa Barbara, CA, USA) operated in a contact mode. The cantilevers used were silicon nitride with a spring constant of 0.6 N/m (Veeco). The AFM was calibrated using a 3D reference of 200 nm height and 10 μ m pitch (Digital Instruments, Santa Barbara, CA, USA). All images shown are deflection images with scan rates of 2.5 Hz with a resolution of 512 pixels/line. The preparation of bacteria for analysis was performed as described previously.³⁴ Briefly, a 20 μ L drop of control (non-treated) bacteria or bacteria pre-incubated with CSA-13 (0.41 mg/L) or LL37 (4.76 mg/L) was applied to cleaved mica (SPI Supplies, West Chester, PA, USA) that had been subjected to 0.1 mg/mL polylysine and left to dry. After 10 min of incubation of the bacterial suspension on the polylysine-covered mica, the sample was rinsed with deionized water and analysed in a wet environment or dried with nitrogen gas for analysis in air.

Evaluation of rhDNase I activity

The enzymatic activity of rhDNase I was evaluated by adding 30 mg/L rhDNase I into solutions of 0.01 mg/mL DNA without (control) or with different concentrations of CSA-13 (0.164–1.64 mg/L) or LL37 (2.38–9.52 mg/L). The activity was obtained from optical density measurements of the samples with subtraction of light scattering (OD₂₆₀–OD₃₂₀). When DNase digests DNA, the optical density at 260 nm increases, and the initial rate of change in OD₂₆₀ is proportional to the DNase activity.

Sputum samples

CF sputum samples were collected by spontaneous expectoration from patients attending the Adult Cystic Fibrosis Center, University of Pennsylvania Health System at Presbyterian Hospital, after obtaining an IRB exemption from the Office of Regulatory Affairs, Institutional Review Board (IRB protocol 803255). For all experiments, samples were diluted with PBS (1:10), vortexed and treated for 1 h with LL37 (0.952–238 mg/L), CSA-13 (0.164–41 mg/L), rhDNase I (10 mg/L) or a combination of CSA-13 with rhDNase I.

Red blood cells haemolysis

The haemolytic activity of the peptides and CSA-13 against human red blood cells (RBCs) was tested as described previously.²⁵ RBCs prepared from fresh blood suspended in PBS (haematocrit ~5%) were mixed with antibacterial agents and incubated for 1 h at 37°C. The samples were then centrifuged at 1300 g for 10 min for haemoglobin release analysis. Relative haemoglobin concentration in supernatants was measured at 540 nm absorbance. Hundred per cent haemolysis was taken from samples to which 1% Triton X-100 was added.

Results

Aggregation of F-actin and DNA by antibacterial agents

The formation of DNA (0.3 mg/mL) and F-actin (0.1 mg/mL) aggregates was detected by changes in light scattering. Aggregation results from the complex formation of antibacterial peptides with DNA and F-actin, which decreases the bactericidal activity of peptides. The polyelectrolyte bundling activities of the antibacterial agents LL37, WLBU2, HB71 and CSA-13 differ over a wide range (Figure 2). CSA-13 has the least effect on DNA or actin aggregation. The extent of aggregation did not depend on the nature of the pH buffer or other monovalent ions. When the concentrations of antibacterial agents are kept constant, their bundling abilities are the same in HEPES and PBS (up and downward pointing open triangles in Figure 2a and other data not shown).

The minimal bundling concentrations of LL37 and WLBU2 are similar to the concentrations necessary to ensure effective bacteria killing (Figure 3), but HB71 and CSA-13 formed aggregates only at much higher concentrations. On a weight basis, approximately 10 times more CSA was needed to produce the same degree of aggregation as caused by LL37 or WLBU2. On a molar basis, the differences are even greater. These results suggest that HB71 and CSA-13 may be relatively resistant to inactivation by negatively charged polyanions.

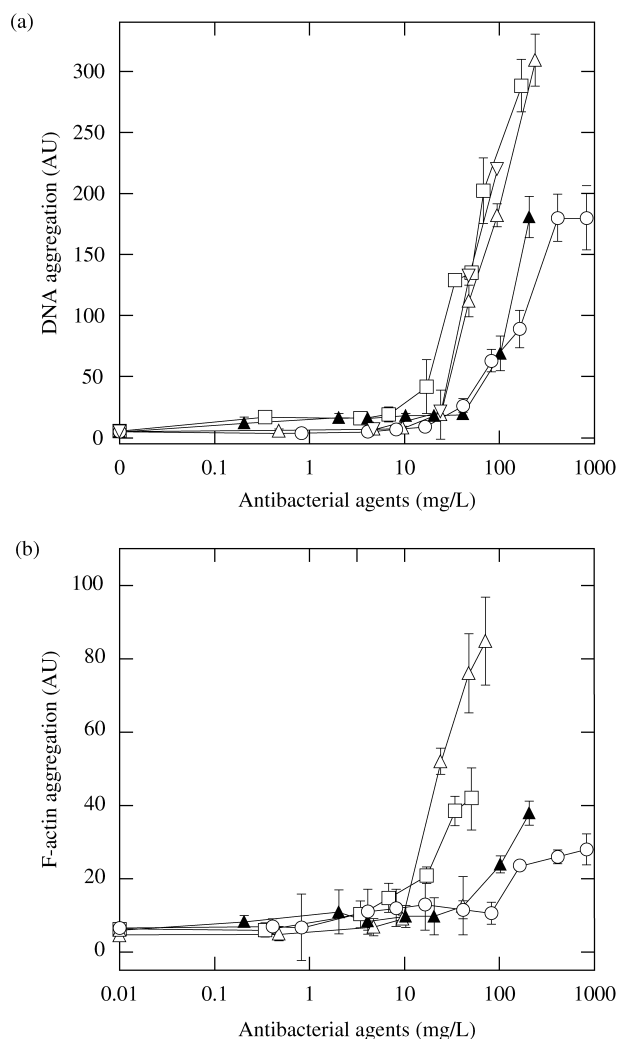


Figure 2. DNA (a) and F-actin (b) bundle formation (in 150 mM NaCl, 5 mM HEPES, 0.2 mM CaCl₂, 0.12 mM MgCl₂, pH 7.35) induced by LL37 (open triangles up), WLBU2 (open squares) and HB71 (filled triangles up) peptides and ceragenin CSA-13 (circles). In (a), open triangles down represent DNA bundle formation in PBS buffer after LL37 addition. Error bars represent standard deviations from three to six measurements.

Inhibition of bactericidal activity by LPS and lipoteichoic acid

By using a conventional bacteria killing assay for *P. aeruginosa* (PAO1), in which bacteria are incubated with antimicrobials for 1 h and then plated onto agar plates (live bacteria are then quantified after an overnight incubation), we compared antimicrobial activities of the LL37, WLBU2 and HB71 peptides with CSA-13 and after their pre-incubation with LPS, LTA or an extract of BLs (Figure 3). All tested antibacterial agents at concentrations in the range of 0.41–10.25 mg/L kill all PAO1 bacteria during a 1 h incubation period (Figure 3).

Previous reports suggest that LPS can interfere with the antimicrobial activity of cathelicidin-LL37 derivatives.^{19,35} Similarly, we observed that the activities of LL37, WLBU2, HB71 and CSA-13 were inhibited by LPS or LTA (Figure 3). As discussed previously,²³ this inhibition is consistent with the possibility that CSA-13 targets bacterial wall LPS and LTA to bind bacteria.²⁷

Under the same conditions but in the presence of BLs rather than LPS or LTA, the value of cfu was much less affected (Figure 3).

The interaction of antimicrobial factors with LPS suggests that they may also alter the effects of LPS on the host inflammatory system. To test this hypothesis, we measured the extent of LPS-mediated NF- κ B translocation to the nuclei of HAECs in the presence of LL37 and CSA-13 (Figure 4). Both CSA-13 and LL37 inhibit the ability of LPS to trigger NF- κ B translocation. On a mass basis, CSA-13 is 5–10 times more potent, with >80% inhibition at a concentration of 1.6 mg/L. This effect suggests that in addition to bactericidal activities, the antibacterial agents LL37 and CSA-13 can also decrease the ability of bacterial products to activate systemic inflammation.³⁶ The ability of CSA-13 and LL37 to prevent LPS-induced cytoplasm to nucleoplasm translocation of NF- κ B was also observed in experiments performed in the presence of 2% human serum (data not shown).

Resistance of CSA-13 to inactivation by DNA and F-actin

The bactericidal activities of LL37, WLBU2 and HB71 are compromised by DNA or F-actin. These polyanions inhibit the antibacterial activity of cationic peptides more strongly than they inhibit CSA-13 (Figure 5), but they do not affect bacterial growth by themselves (data not shown). Evaluation of MIC values confirmed that CSA-13 maintains its high antibacterial activity in the presence of DNA and F-actin (Table 1). Cationic antibacterial peptide activity was also reported to decrease in the presence of millimolar concentrations of divalent cations or blood serum and when bacterial growth sources are available.^{18,19} The ability of CSA-13 to kill bacteria, similar to that of the synthetic peptides WLBU2 and HB71 (Figure 6), was lowered by human serum and 2 mM MgCl₂. But in both cases, CSA-13 was more resistant to inactivation, with <5 mg/L CSA-13 required for 90% killing when compared with over 100 mg/L for LL37. Glucose (5 mM) had little effect on the antibacterial activity of these agents.

AFM image of bacteria damage

Analysis of AFM images of PAO1 bacteria (Figure 7) subjected to CSA-13 and LL37 reveals changes in surface morphology that indicate bacterial wall damage. AFM height image line scans (insets of Figure 7 right-hand panels) across the bacterial membrane reveal a striking increase in membrane roughness for the samples treated with CSA-13 and LL37 when compared with the control. This observation is consistent with previous TEM images showing CSA membrane activity.²⁸ A comparison of the effects of CSA-13 and LL37 suggests that these structurally distinct molecules may share some aspects of the mechanisms of action that cause disruption of membrane integrity.^{37,38}

rhDNase I activity

To test whether ceragenin CSA-13 or LL37 peptide association with DNA affects the function of rhDNase I, we evaluated DNase enzymatic activity in samples containing DNA with different concentrations of CSA-13 and LL37 (Figure 8). The rhDNase I activity decreased ~10% and 50% when its substrate was present together with bactericidal concentrations of CSA-13 or LL37, respectively, and in the case of LL37, this decrease

Activity of CSA-13 in CF sputum

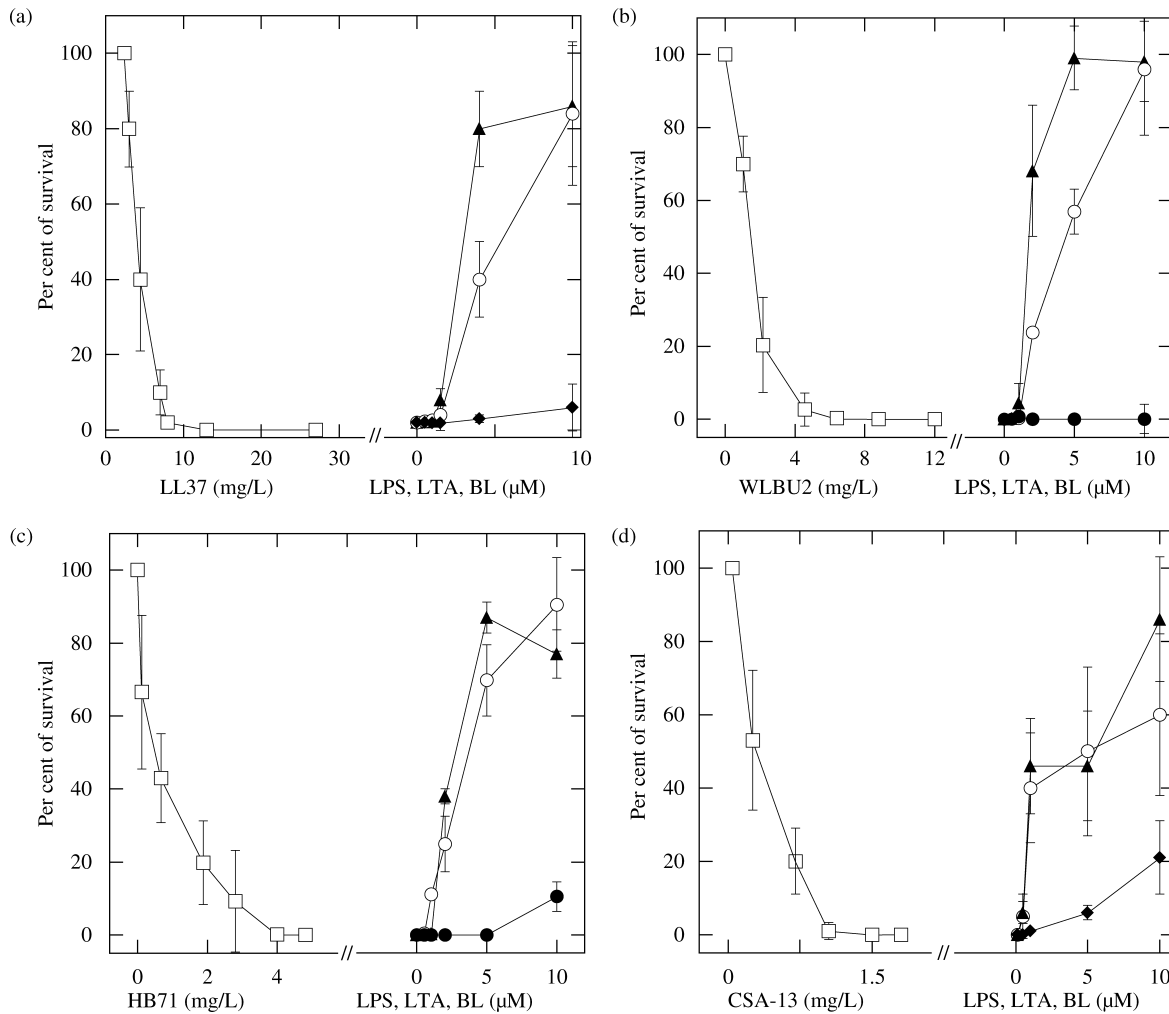


Figure 3. Bactericidal activity of LL37 (a), WLBU2 (b), HB71 (c) and CSA-13 (d) against PAO1 bacteria (in PBS: 150 mM NaCl, 2 mM KCl, 10 mM Na₂HPO₄, 5 mM NaH₂PO₄, pH 7.35), by themselves (squares), and in the presence of LPS (triangles), LTA (circles) and an extract of BLs (diamonds). Effect of LPS, LTA and BL was evaluated with a constant concentration of LL37, WLBU2, HB71 and CSA-13 (1 μM of each corresponding to 4.76, 3.4, 2.05 and 0.82 mg/L, respectively). As the molecular weight of LPS is heterogeneous (10–100 kDa), we have used the lowest range of reported LPS MW in buffer without divalent cations (our stock solution of LPS was made in H₂O) to calculate LPS molar concentration. Error bars represent standard deviations from four measurements.

was statistically significant. These data suggest that CSA-13 may have a much lower impact on rhDNase I activity than agents currently used in CF treatment.

Bacteria load of CF sputa

As shown in Figure 9, CSA-13 decreases bacterial outgrowth from CF sputum more potently than an equal concentration of LL37, WLBU2 or HB71. A 90% decrease in bacterial outgrowth was observed after addition of 20 mg/L CSA-13, compared with only a 60% decrease with 250 μM LL37. The extent of bacterial growth inhibition by 8.2 mg/L CSA-13 in combination with rhDNase I (10 mg/L) was greater than that achieved by 8.2 mg/L CSA-13 alone. The lack of effect of rhDNase I on PAO1 bacteria growth (data not shown) and the observed ~20% decrease of bacterial outgrowth after rhDNase I addition to CF sputa samples show that its addition can lessen bacterial growth by a mechanism other than direct bacterial killing, which is consistent with its ability to liberate endogenous antibacterial activities within CF sputum.¹⁴

Effect of CSA-13 on RBCs

Leakage of haemoglobin from RBCs provides an independent quantitative assay for the extent of membrane permeabilization. By evaluating haemoglobin release from RBCs (Figure 10), we show no significant membrane permeabilization by LL37, HB71 or CSA-13 at concentrations sufficient to kill bacteria with high efficiency (Figure 2). In contrast to RBC tolerance of LL37, HB71 and CSA-13, RBCs were significantly damaged by the WLBU2 peptide and melittin, which is known to cause haemoglobin leakage through pore formation. With increasing concentration (>10 mg/L), CSA-13 caused more RBC lysis than did LL37 and HB71 peptides; however, it was able to decrease the CF sputum bacterial load to the same extent that LL37 did at a concentration of 230 mg/L (Figure 9). The ability to disrupt host cell membrane at concentrations much higher than those required to kill bacteria supports the hypothesis that LL37 and CSA-13 may function by the ‘carpet-like’ mechanism,³⁷ rather than by pore formation.

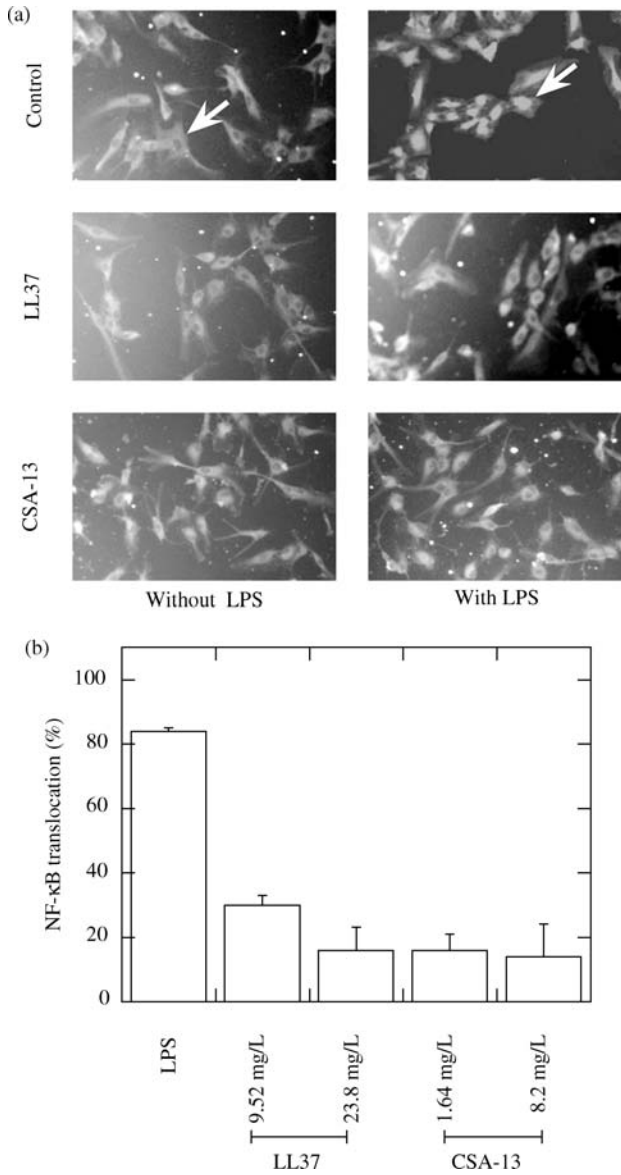


Figure 4. LPS-induced (0.1 mg/L) translocation of NF-κB from cytoplasm to nucleoplasm of HAEC (in EBM-2 with supplements) is inhibited by LL37 (47.6 mg/L) and CSA-13 (8.2 mg/L) (arrows indicate nuclei without and with bright spots in control and LPS-treated sample, respectively). Data from one representative experiment are shown (a). Quantification of NF-κB translocation to the nucleus in HAEC after LPS, LPS + LL37 or LPS + CSA-13 treatment (b). Error bars represent standard deviations from three experiments.

Discussion

Membrane-active cationic antimicrobial peptides and some cationic steroids, or ceragenins, offer alternatives as antimicrobial agents that can be used to combat bacterial drug resistance often associated with chronic infection. The potency of mammalian-derived peptides to kill bacteria is only optimal under specific, often non-physiological, conditions, which may generate multiple obstacles to design a therapeutically suitable peptide.¹⁸ In CF airways, host antibacterial peptide function was found to be suppressed by DNA and F-actin as a result of electrostatic

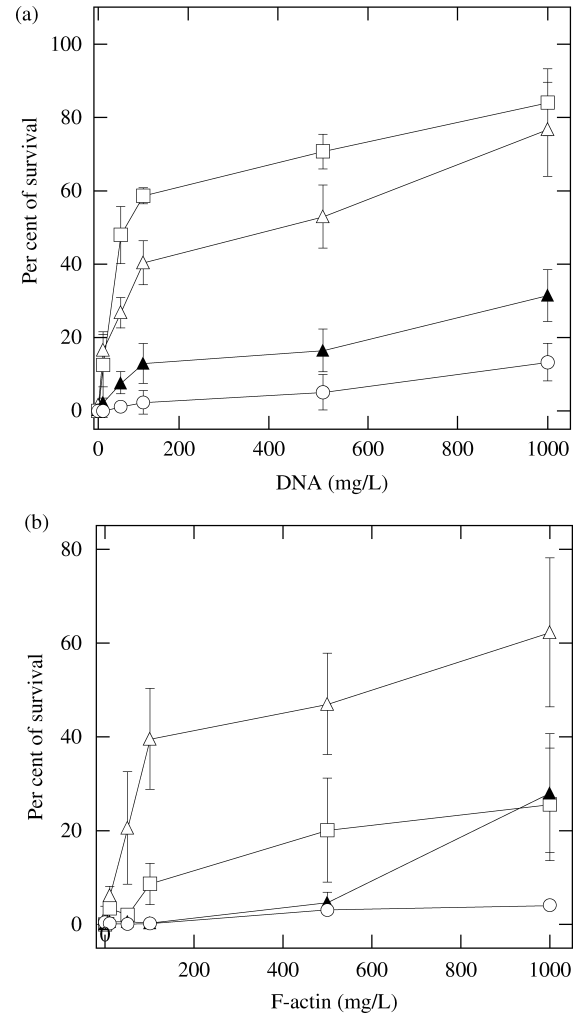


Figure 5. DNA (a) and F-actin (b) inhibit bactericidal activities of antibacterial agents against PAO1 bacteria in a concentration-dependent manner (experiments performed in PBS: 150 mM NaCl, 2 mM KCl, 10 mM Na₂HPO₄, 5 mM NaH₂PO₄, pH 7.35). LL37 (open triangles up), WLB2 (open squares), HB71 (filled triangles up) and CSA-13 (circles) (9.52, 6.8, 4.1 and 1.64 mg/L, respectively). Error bars represent standard deviations from four measurements.

interactions between the peptide and DNA or F-actin.¹⁴ According to the present study, synthetic CSA-13 offers an advantage over the natural LL37 and synthetic WLB2 and HB71 peptides in killing *P. aeruginosa* bacteria in the presence

Table 1. Activity of tested antibacterial agents against PAO1 bacteria

	MIC (mg/L)			
	LL37	WLB2	HB71	CSA-13
MH, 2 mM MgCl ₂	50	12.5	12.5	3.125
MH, 2 mM MgCl ₂ , 1 mg/mL DNA	>200	>200	100	12.5
MH, 2 mM MgCl ₂ , 1 mg/mL F-actin	>200	100	25	6.25

Activity of CSA-13 in CF sputum

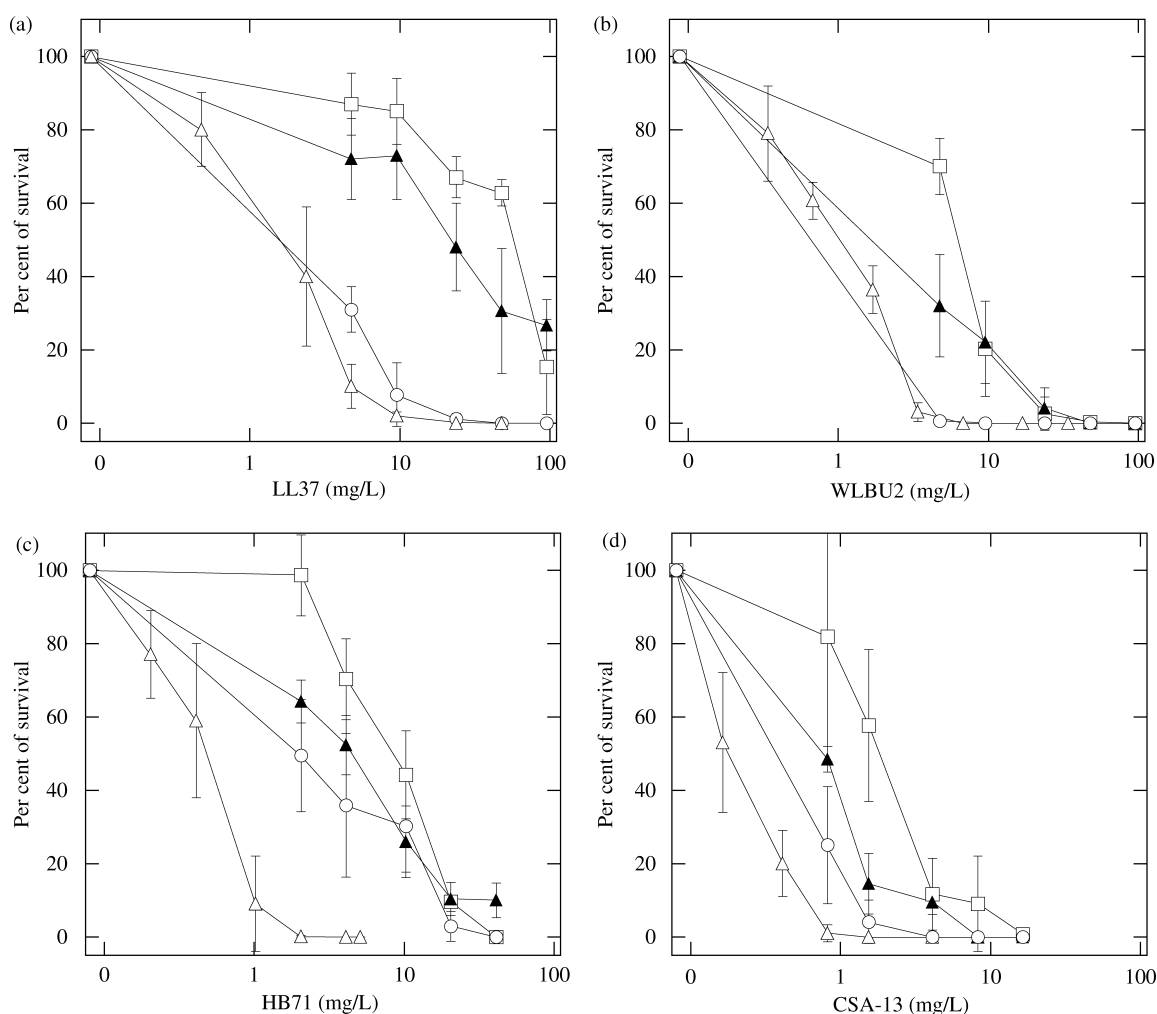


Figure 6. Bactericidal activity of LL37 (a), WLBU2 (b), HB71 (c) and CSA-13 (d) in PBS (150 mM NaCl, 2 mM KCl, 10 mM Na₂HPO₄, 5 mM NaH₂PO₄, pH 7.35) (open triangles up), PBS containing 10% dehydrated human serum (open squares), 150 mM NaCl, 5 mM HEPES with 2 mM MgCl₂ (filled triangles up) and PBS containing 5 mM glucose (circles). Error bars represent standard deviations from three to four experiments.

of abundant polyanionic components of CF sputum. The differences in molecular structure (Figure 1) between CSA-13 and cationic antibacterial peptides may help to explain why CSA-13 activity is less compromised by negatively charged DNA and F-actin, thereby allowing it to interact with exposed charges on bacterial membranes.

CSA-13 is a smaller molecule than LL37, with lower positive charge and different charge density. These features may explain the observed differences between the ability of CSA-13 and peptides tested here (LL37, WLBU2 and HB71) to form aggregates when added to polyanionic biopolymers. A higher molar concentration of CSA-13 is required to observe its interaction with DNA or F-actin and this CSA-13 concentration corresponds to a concentration range of natural polyamines (spermine and spermidine) required to induce F-actin bundle formation.^{39,40} This result suggests that there exist similarities in behaviour of the positively charged face of CSA-13 and natural polyamines.

The ability of CSA-13 to maintain its activity in a CF sputum environment indicates that molecules from the CSA family might avoid CF airway fluid factors that inhibit the function of endogenous antimicrobials. The non-peptide nature of

CSA-13 may also enhance its stability and reduce clearance kinetics to a level similar to that of conventional antibiotics. Even though they function by a different mechanism from antibacterial peptides and cationic steroids, many conventional antibiotics such as tobramycin are also cationic and can be inhibited by the same factors that limit antibiotic peptides.⁴¹ The potency of CSA-13 even in CF sputum suggests that it has potential for therapeutic use. Current therapies using nebulized tobramycin are designed to deliver >128 mg/L to the sputum of CF patients, and quantification of tobramycin incorporation into sputum after aerosolized delivery of 300 mg showed peak mean concentrations of 687 μ g of tobramycin per gram of sputum.⁴² By comparison, Figure 6 shows that 20 mg/L CSA-13 killed 90% of bacteria in a sputum sample diluted in 9 volumes of buffer, corresponding to \sim 200 μ g of CSA-13 in 1 g of sputum.

The additive effect of CSA-13 (8.2 mg/L) with recombinant human DNase I (10 mg/L) to decrease bacteria outgrowth from CF samples indicates that ceragenins can overcome this limitation of aminoglycoside antibiotics and strongly confirms our previous observation that factors depolymerizing DNA and

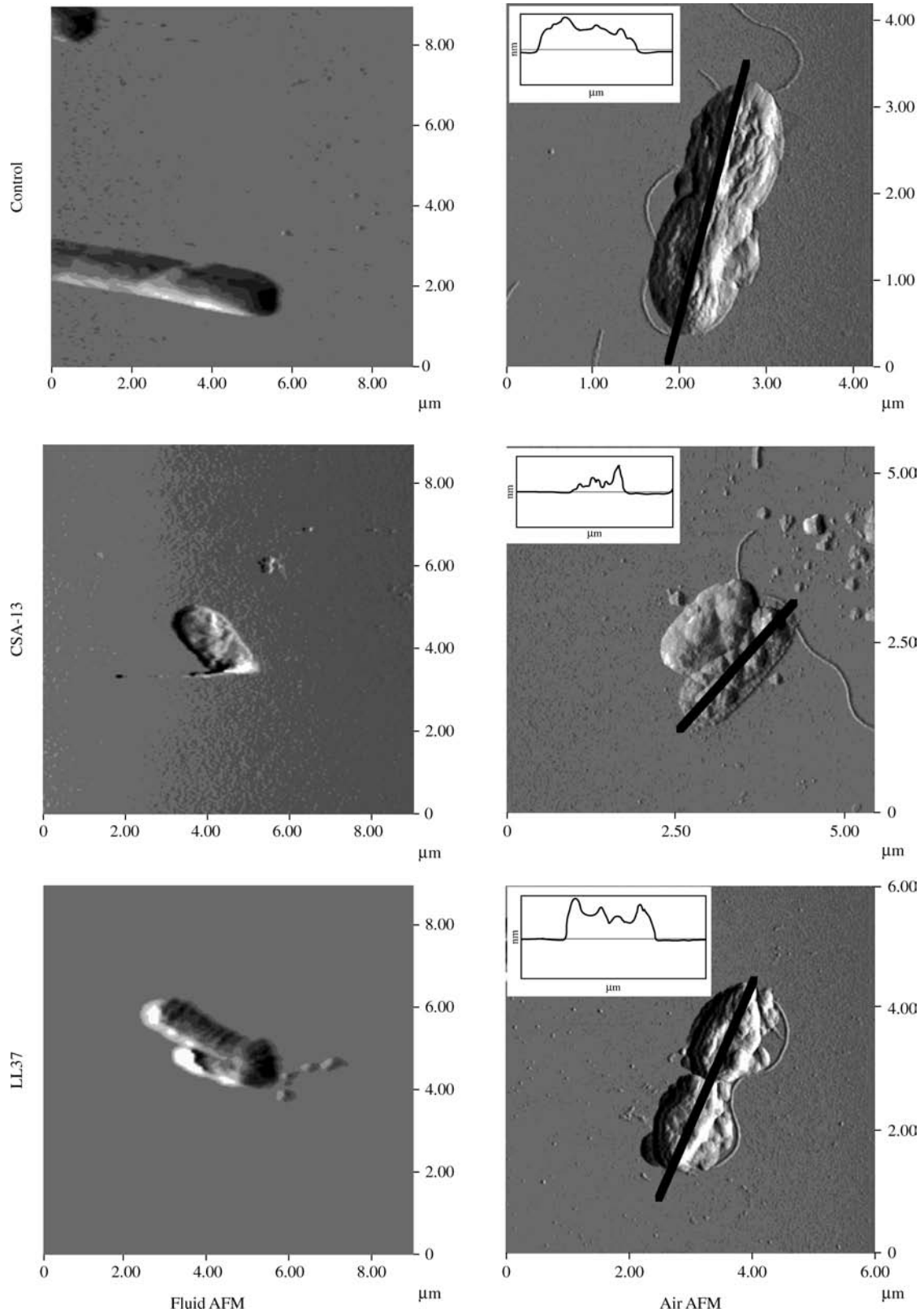


Figure 7. AFM deflection images in a fluid environment (left-hand panels) and in an air environment (right-hand panels). The images depict the surface morphology of PAO1 bacteria in a control sample (upper row) and after treatment in PBS buffer (150 mM NaCl, 2 mM KCl, 10 mM Na₂HPO₄, 5 mM NaH₂PO₄, pH 7.35) with CSA-13 (middle row) or LL37 (lower row). Each inset graph displays a line scan extracted from AFM height images (data not shown) for a more quantitative look at the surface morphological variances; y-axis (height) scales are the same in each line scan. Data from one representative experiment performed in triplicate are shown.

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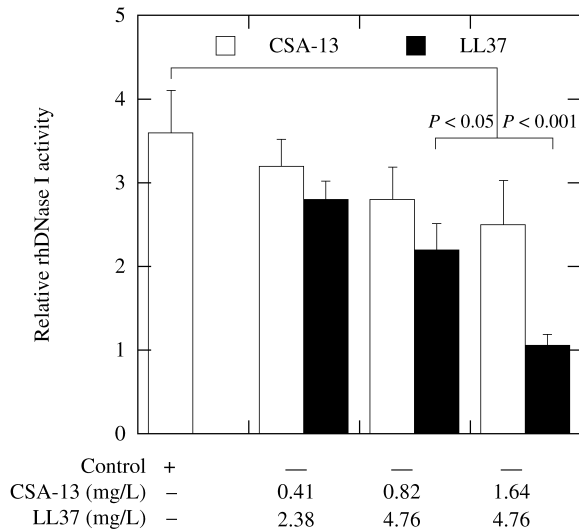


Figure 8. Pulmozyme (rhDNase I) activity evaluated in DNA samples (150 mM NaCl, 5 mM HEPES, 2 mM CaCl₂, pH 7.35) incubated with various concentrations of CSA-13 (white bars) and LL37 (black bars). Each data point represents the mean of four experiments. Differences between mean data in this experiment were evaluated by Student's *t*-test, with $P < 0.05$ being taken as the level of significance.

F-actin, such as rhDNase I and gelsolin, may release the cationic antibacterial molecules from CF bundles.⁷

Microscopic evaluation by AFM of PAO1 bacteria treated with LL37 and CSA-13 reveals that under their action, bacterial membranes lose their integrity, causing bleb formation and bacterial body disintegration. In previous studies, the interaction of CSA-13 or LL37 with anionic charges on bacterial membranes followed by their insertion was found to cause membrane damage by a detergent-like mechanism.^{43,44}

Unlike its resistance to inhibition by F-actin and DNA, CSA-13 is affected by the bacterial wall components LTA and LPS similar to LL37, WLBU2 and HB71 peptides, but a potentially beneficial consequence of this interaction is the suppression of inflammation that can exacerbate the pathologic state in CF and other infections. Antibacterial agents that function by targeting LPS or LTA may also suppress the immunostimulatory effect of these bacterial wall components on the host.⁴⁵ Delivery of LPS from external fluids to the cell membrane and ultimately to Toll-like receptors (TLRs) may involve a number of external proteins including CD14, LPS-binding protein and other factors.⁴⁶ TLR activation results in NF- κ B translocation from cytoplasm to nucleoplasm. In untreated primary HAEC, NF- κ B was located in the cytoplasm (Figure 4) and activation by LPS resulted in its translocation to the nucleus, similar to the localization induced by TNF- α . The finding that LL37 and CSA-13 prevent LPS-mediated NF- κ B translocation suggests that binding of these antibacterial molecules to LPS prevents LPS interaction with TLRs, and this effect may contribute to anti-inflammatory functions of cationic antibacterial agents.

With regard to haemolytic activity, previous research has shown that many antibacterial peptides lyse RBCs, but the required lytic concentration is usually higher than their bactericidal concentration.⁴⁷ A detectable increase in haemoglobin release after CSA-13 addition to RBC suspensions occurs at a concentration 10 times higher than its effective concentration to

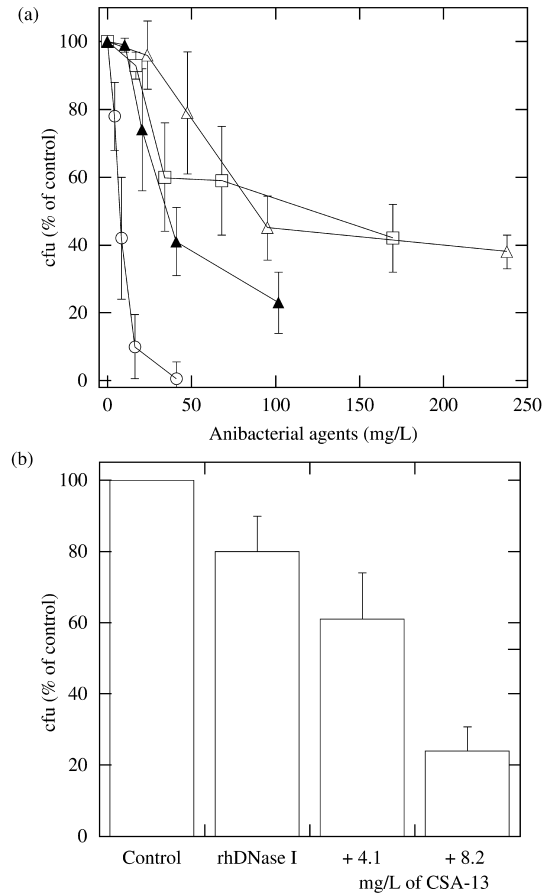


Figure 9. (a) cfu of CF sputa after treatment with various concentrations of LL37 (open triangles up), WLBU2 (open squares), HB71 (filled triangles up) and CSA-13 (circles). (b) cfu of CF sputa after treatment with rhDNase I (10 mg/L) or its combination with CSA-13 (+4.1 or +8.2 mg/L, respectively). Error bars represent standard deviations from four different CF sputa samples obtained from patients chronically infected with *P. aeruginosa*.

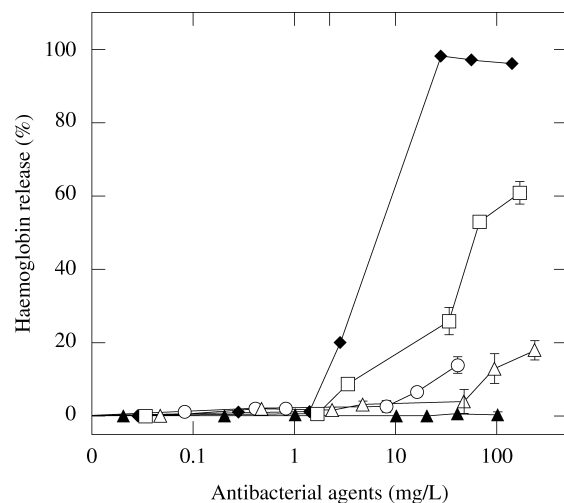


Figure 10. Haemoglobin release from RBCs suspended in PBS after addition of LL37 (open triangles up), WLBU2 (open squares), HB71 (filled triangles up), CSA-13 (circles) and melittin (diamonds), followed by incubation for 1 h at 37°C. Error bars represent standard deviations from four measurements.

kill bacteria, suggesting that it may function similar to antibacterial peptides.

In conclusion, we show that CSA-13, a member of the ceragenin family, like natural LL37 or synthetic WLBU2 and HB71 peptides targets the bacterial wall through interactions with LPS and LTA. The ability of CSA-13 to maintain its bactericidal activity in the presence of negatively charged F-actin and DNA and the observed additive effect of CSA-13 with rhDNase I suggest that this synthetic antibiotic has potential advantages in treating chronic infection in CF airways.

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Transparency declarations

None to declare.

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