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PepBiotics, novel cathelicidin-inspired antimicrobials to fight pulmonary bacterial infections

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ABSTRACT

Background: Antimicrobial peptides are considered potential alternatives to antibiotics. Here we describe the antibacterial properties of a family of novel cathelicidin-related (CR-) peptides, which we named PepBiotics, against bacteria typically present in cystic fibrosis (CF) patients.
Methods: Broth dilution assays were used to determine antibacterial activity of PepBiotics under physiological conditions, as well as development of bacterial resistance against these peptides. Toxicity was tested in mice and cell cultures while molecular interactions of PepBiotics with bacterial membrane components was determined using CD, ITC and LPS/LTA induced macrophage studies.
Results: A relatively small number of PepBiotics remained highly antibacterial against CF-related respiratory pathogens *Pseudomonas aeruginosa* and *Staphylococcus aureus*, at high ionic strength and low pH. Interestingly, these PepBiotics also prevented LPS/LTA induced activation of macrophages and was shown to be non-toxic to primary human nasal epithelial cells. Furthermore, both *P. aeruginosa* and *S. aureus* were unable to induce resistance against CR-163 and CR-172, two PepBiotics selected for their excellent antimicrobial and immunomodulatory properties. Toxicity studies in mice indicated that intratracheal administration of CR-163 was well tolerated in vivo. Finally, interaction of CR-163 with bacterial-type anionic membranes but not with mammalian-type (zwitterionic lipid) membranes was confirmed using ITC and ^{31}P solid state NMR.
Conclusions: PepBiotics are a promising novel class of highly active antimicrobial peptides, of which CR-163 showed the most potential for treatment of clinically relevant (CF-) pathogens in physiological conditions.
General significance: These observations emphasize the therapeutic potential of PepBiotics against CF-related bacterial respiratory infections.

1. Introduction

Antimicrobial peptides (AMPs) are considered potential alternatives to antibiotics for more effective and resistance-proof treatment of bacterial infections [1]. Besides direct antimicrobial activity against bacteria, fungi and viruses, many AMPs also display a multitude of immunomodulatory functions, including neutralization of bacterial compounds such as LPS and LTA [2,3]. In the last decade the number of studies focusing on discovery of new or optimized AMPs, especially with respect to their microbicidal activity, has grown exponentially [4,5].

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One strategy to obtain optimized lead structures and lead sequences is by rational design of peptides to enhance their antimicrobial activity [5–8]. These studies showed promising results based on a few general guidelines for peptide design: increased charge results in higher activity [6], hydrophobicity should be around 50% [7] and peptides should have amphipathic properties [8]. Unfortunately, these parameters also result in increased toxicity (often measured as hemolytic activity) [9] so the challenge is to find a therapeutic window where antimicrobial and hemolytic concentrations of peptide are sufficiently diverged for potential therapeutic use [5].

Despite the rapidly expanding set of known natural and (re)designed AMPs, it has become clear that the Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) of novel AMPs will likely be in the same order of magnitude as most natural AMPs [10]. This suggests that the search for clinically beneficial AMPs should not be solely based on finding the lowest MIC possible. Instead, designer peptides should be tested for specific clinical purposes. This could include specificity of AMPs for certain bacterial strains, but equally important is the consideration of the environmental conditions in which AMPs will be used therapeutically. The major pitfall of the current MIC test for determination of antimicrobial activity is that this is performed under non-physiological conditions [11]. Relatively simple parameters such as the presence of cations, ionic strength and pH can have dramatic effects on the activity of AMPs [2,12–14]. This implies that AMPs measured as highly active *in vitro*, may show little or no activity *in vivo* [15–17]. In addition, strain differences within bacterial species can also result in differences of susceptibility for AMPs [17–19].

Considering the above, our group has designed a large set of novel AMPs designated PepBiotics. These Cathelicidin-Related (CR) peptides (filed for patent protection: PCT/EP2018/060402; “antimicrobial peptides and their use”) are identified by a core sequence of 12 amino acids, RRWVQRWIRRWR, obtained through several iterative rounds of optimization for antimicrobial activity under standard assay conditions, and PepBiotics can now be screened as novel therapeutics for specific diseases or specific (bacterial) infections. One such disease is Cystic Fibrosis (CF), in which PepBiotics could target the most common pathogens *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Treatment of bacterial infections in the CF lung is currently still heavily dependent on the use of antibiotics, and therefore the worldwide increase in bacterial resistance to antibiotics is a severe threat to treatment options [20]. Since CF is characterized by high salt concentrations and a lowered pH [21,22], PepBiotics were tested in the current study under these conditions to determine their potential as novel therapeutics to treat CF disease. The study identifies a small group of active peptides and eventually focuses on PepBiotic CR-163 as the most promising AMP for this specific application.

2. Methods

2.1. Peptides

All peptides (Table 1) were synthesized by China Peptides (Shanghai, China) using Fmoc-chemistry. The peptides were purified by reverse phase high-performance liquid chromatography to a purity of >95%. Peptides were dissolved in sterilized H_2O at a stock concentration of 1600 μM and stored in aliquots at −80 °C.

2.2. Bacterial strains

Eight *Pseudomonas aeruginosa* (*P. aeruginosa*) and eight *Staphylococcus aureus* (*S. aureus*) strains were used in this study (all donated by University Medical Centre Utrecht (UMCU), Department of Medical Microbiology, Utrecht, The Netherlands). All strains were clinical isolates obtained from sputum samples of CF-patients except for two *P. aeruginosa* strains obtained, with formal consent, from blood samples of CF-patients (see Table S1).

2.3. Antimicrobial activity

Antimicrobial activity of PepBiotics was tested against *P. aeruginosa* and *S. aureus* strains by colony count (track dilution) assays. Bacteria were maintained in Mueller Hinton Broth (MHB, Oxoid Limited, Hampshire, UK) at 37 °C and grown to mid-logarithmic phase before testing. Bacteria were diluted to 2×10^6 CFU/ml in MHB or MHB adjusted for NaCl concentration or pH (where indicated), and 25 μl was mixed with 25 μl of peptide (0–40 μM) in polypropylene round-bottom (96 wells) plates and incubated for 3 h at 37 °C. Subsequently, viable bacteria were enumerated by preparing a series of 10-fold dilutions, of which 15 μl was added to agar plates. After incubation overnight at 37 °C, colonies were counted. MBC was defined as the concentration where the inoculum was reduced to $\leq 1.3 \times 10^2$ CFU/ml (corresponding to ≤2 colonies on the agar plate).

2.4. Peptide-mediated LPS and LTA neutralization

Murine RAW 264.7 macrophages (ATCC TIB-71) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in DMEM (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% FCS (Bodinco B.V., Alkmaar, The Netherlands) at 37 °C, 5.0% CO_2. At the start of an experiment 5×10^4 RAW cells were seeded in 96-well plates for adherence overnight prior to stimulation. The following day, PepBiotics (1, 5 and 20 μM) and 50 ng/ml *P. aeruginosa* LPS (Sigma-Aldrich, Zwijndrecht, The Netherlands) or 1

Table 1
Cathelicidin-related peptides used in this study: sequence and characteristics. The patented core sequence for antimicrobial activity of PepBiotics is indicated in bold.

Name	Sequence	AA/charge	Mw
CR-156	**RRWVQRWIRRWR**PKV	15/8	2177.61
CR-160	**RRWVQRWIRRWR**KV	14/8	2080.50
CR-161	**RRWVQRWIRRWR**KPV	15/8	2177.61
CR-162	**RRWVQRWIRRWR**PWV	15/7	2235.65
CR-163	**RRWVQRWIRRWR**KWV	15/8	2266.71
CR-164	RRWVQRWIRWVQRPV	15/6	2120.51
CR-165	RRWVQRWIRWVQRWVR	16/7	2365.79
CR-166	**RRWVQRWIRRWR**PK	14/8	2078.48
CR-167	**RRWVQRWIRRWR**PKW	15/8	2264.69
CR-168	RRWVRRWIRRWRPKV	15/9	2205.67
CR-169	**RRWVQRWIRRWR**PKVAAARRWV	22/10	2988.56
CR-170	**RRWVQRWIRRWR**PKRIVQRIKDFLRNLV	28/11	3830.60
CR-171	RRWGRFLRKIRRFRPKFKRIVQRIKDFLRNLV	32 /14	4255.18
CR-172	**RRWVQRWIRRWR**PKVAAA**RRWVQRWIRRWR**PKV	33 /15	4551.43
CR-173	**RRWVQRWIRRWR**KVAAA**RRWVQRWIRRWR**PKV	32/15	4454.31
CR-184	APKAM**RRWVQRWIRRWR**PKVFQVTGSSA	28/9	3454.08

μg/ml *S. aureus* LTA (Bioconnect B.V., Huissen, The Netherlands) were premixed in DMEM/FCS, immediately subjected to the RAW 264.7 cells and incubated for 24 h. Subsequently, the supernatant was used to determine NO production using the Griess assay as described [23].

2.5. Cytotoxicity

In vitro cytotoxicity of PepBiotics was tested using primary human nasal epithelial cells (HNECs). Cells were isolated and cultivated to confluency using Bronchial Epithelical Cell Medium (BEPC, Sciencell Research Laboratories, Sanbio, Uden, Netherlands) as described [24]. HNECs were cultured in 96-well plates (10^3 cells/well) until confluent and subsequently exposed to 5 μM PepBiotics for 3 h at 37 °C. Metabolic activity was assessed by replacing the peptide-containing medium with fresh BEPC containing 10% (*v*/v) WST-1 reagent. After 30 min the color-change of the medium (indicative for metabolic activity) was measured for absorbance at 450 nm on a FLUOstar Omega microplate reader (BMG Labtech GmbH, Ortenberg, Germany) and was corrected for absorbance at 630 nm.

2.6. In vivo tolerance

Male C57Bl/6 mice (Charles River, Sherbrooke, Qc, Canada), weighing 22–30 g, were randomized to one of seven treatment groups (7 animals/group) and injected with 50 μL of: 1) saline, 2) 20 μM (88 μg/kg) CR-163, 3) 100 μM CR-163 (439 μg/kg), 4) 500 μM CR-163 (2.2 mg/kg), 5) 20 μM CR-173 (176 μg/kg), 6) 100 μM CR-173 (882 μg/kg) 7) 500 μM CR-173 4,4 mg/kg). All peptides were dissolved in saline. Each animal was anesthetized by intraperitoneal injection of a ketamine (130 mg/kg BW) and dexmedetomidine (0.5 mg/kg BW), and then intubated using a 20 G catheter, with the aid of a fiber-optic stylet (BioLite intubation system for small rodents, BioTex, Inc., Houston, Texas, USA). Once intubated, mice received the 50 μL intratracheal instillation according to its randomization. Mice were subsequently extubated and injected with the reversal agent for dexmedetomidine, Antisedan, and allowed to breathe spontaneously for the following four hours. During this period, mice were monitored using a health assessment scoring protocol in which general health, feces (diarrhea), respiratory effort, behavior/activity, and alertness, were assessed on a scale of 0–3, and animals were euthanized if either an animal received a score of 3 or a cumulative of 10. After 4 h, the mice were euthanized by IP injection of sodium pentobarbital and dissection of the descending aorta. After performing a tracheostomy and exposing the lungs, animals were connected to the FlexiVent (SCIREQ, Montreal, Quebec, Canada) for *ex vivo* measurements of lung function via various perturbations starting with a deep inflation assessment of inspiratory capacity. Following the deep inflation, a snapshot perturbation, a prime-8, and pressure-volume (PV) curve through controlled stepwise increasing pressure, were performed. These software-controlled perturbations were performed with 10 s intervals of *ex vivo* ventilation [25,26].

Following the measurements of lung mechanics, whole lung lavage fluid was collected using 3 × 1 ml aliquots of sterile saline, as previously reported. The whole lung lavage was immediately centrifuged at 150 x g at 4 °C, and the pellet was collected for differential cell analysis as previously described [27]. The supernatant was used to measure protein within the lavage fluid using a Micro BCA protein assay kit (Pierce, Rockford, Ill., USA), according to the manufacturer's instructions. Supernatant was also utilized to measure lavage concentrations of three inflammatory mediators, IL-6, TNF-α and IL-8, using an enzyme-linked immunosorbent assay (ELISA) kit (BD Biosciences, San Diego, Calif., USA), according to manufacturer's instructions.

All animal procedures were approved by the Animal Use Subcommittee at the University of Western Ontario, and followed the approved guidelines described by the Canadian Council of Animal Care.

2.7. Development of antimicrobial resistance

The PepBiotics CR-163 and CR-172 were selected for measuring development of resistance in *P. aeruginosa* and *S. aureus*; the antibiotic ciprofloxacin was included as reference. A starting MIC value (defined as no visible growth of bacteria after o/n incubation with PepBiotics) was determined for peptides CR-163, CR-172 and ciprofloxacin against an o/n culture of *P. aeruginosa* (strain 8) and *S. aureus* (strain 21). In 5 ml tubes (Falcon Corning Science Mexico), 0.5 ml of a 20-fold diluted culture was exposed to a concentration range of peptide (0–160 μM) or ciprofloxacin (0–4096 μg/ml) and incubated o/n. The following day the tube with the highest peptide/ciprofloxacin concentration (sub-MIC tubes) still showing visible growth was selected. This culture was diluted 20-fold and subsequently exposed again to a concentration range of peptides and ciprofloxacin. In addition, the culture was diluted, plated on TSA plates, incubated o/n and growing bacteria were identified using MALDI-TOF to check for potential contaminations. After 15 (CR-163) or 19 days (CR-172), the sub-MIC tubes for both bacterial species were grown o/n in MHB and the following day the MIC was determined to check if developed resistance was sustained even in the absence of peptide or ciprofloxacin. In addition, cross-resistance was tested by exposing ciprofloxacin-resistant cultures to PepBiotics and vice versa.

2.8. Amino acid scans of CR-163

CR-163 was used to determine which amino acid residues are vital in generating the antimicrobial activity of the core sequence of PepBiotics. To this end, each individual amino acid of the CR-163 core sequence was replaced by an Ala residue (alanine scan). Activity of the resulting panel of CR-163 mutants was measured by the track dilution assay against PA8 and SA21 using 8 μM peptide. Similarly, an 'aromatic scan' was performed on CR-163 in which three Trp residues present in the CR-163 sequence, were either mono-, di- or tri-substituted for Tyr, Phe or Ile.

2.9. ^{31}P solid state NMR

Unilamellar palmitoyloleoyl-phosphatidylcholine (POPC) and palmitoyloleoyl-phophatidylglycerol (POPG) vesicles were prepared in 5 mM HEPES pH 7.5 and 50 mM NaCl by the extrusion technique using 0.2 μm filters [28]. Phospholipid concentration was determined as inorganic phosphate after treatment with perchloric acid by UV-VIS spectroscopy [29]. CR-163 was added to POPC and POPG vesicle suspensions to a final molar ratio of 1:50 CR-163/POPC or 1:50 CR-163/POPG. Subsequently, vesicles were collected after ultracentrifugation and were spun down in 3.2 mm rotors. Static ^{31}P solid-state NMR spectra were acquired at 500 MHz magnetic field (^1H-frequency) and a sample temperature of 295 K. The resulting ^{31}P powder patterns were apodized with 50 Hz exponential line-broadening and baseline corrected.

2.10. Circular dichroism

CD-spectra were obtained using a Jasco J-810 CD-spectrometer with a quartz cell that had a 1 mm pathlength. For the measurements the samples used in the NMR experiments were diluted to a peptide concentration of 25 μM with the same buffer (5 mM HEPES pH 7.5 and 50 mM NaCl). The spectra were recorded at room temperature from 250 to 190 nm at a 0.2 nm step size with 1-s response time. The spectra were averaged over 4 recordings.

2.11. Isothermal titration calorimetry

Interaction between CR-163 and large unilamellar vesicles consisting of POPC and/or POPG was tested using isothermal titration calorimetry (ITC). All ITC experiments were performed on a Low Volume NanoITC (TA instruments - Waters LLC, New Castle, DE USA). POPC and POPG vesicles were prepared as described above. POPG vesicles were diluted

to 0.15 mg/ml before measurements, POPC vesicles to 1.5 mg/ml. In each experiment, the ITC cell chamber was filled with 169 μl of vesicles, and the syringe was filled with a 50 μl solution of 320 μM CR-163. Titrations were incremental with 2 μl injections at 300 s intervals. Experiments were performed at 37 °C and data were analyzed with the Nano Analyze software (TA instruments - Waters LLC).

2.12. Statistics

Statistical significance was assessed with one-way ANOVA followed by the Dunnett Post-Hoc test in GraphPad Prism software, version 6.02. A p-value of <0.05 was considered statistically significant.

3. Results

3.1. MBC determination and strain-specificity of CR-156

CR-156, a peptide which was shown to have high antibacterial activity in pilot experiments of the iterative development process, was investigated for its antimicrobial potential against CF pathogens *P. aeruginosa* and *S. aureus*. For both bacterial species, eight different CF isolates were tested using increasing concentrations of CR-156 by track dilution assays. Only small differences in susceptibility for CR-156 were observed between individual strains from each species with an MBC of 2.5–5 μM for all *P. aeruginosa* strains and 5–10 μM for all *S. aureus* strains (Table S1).

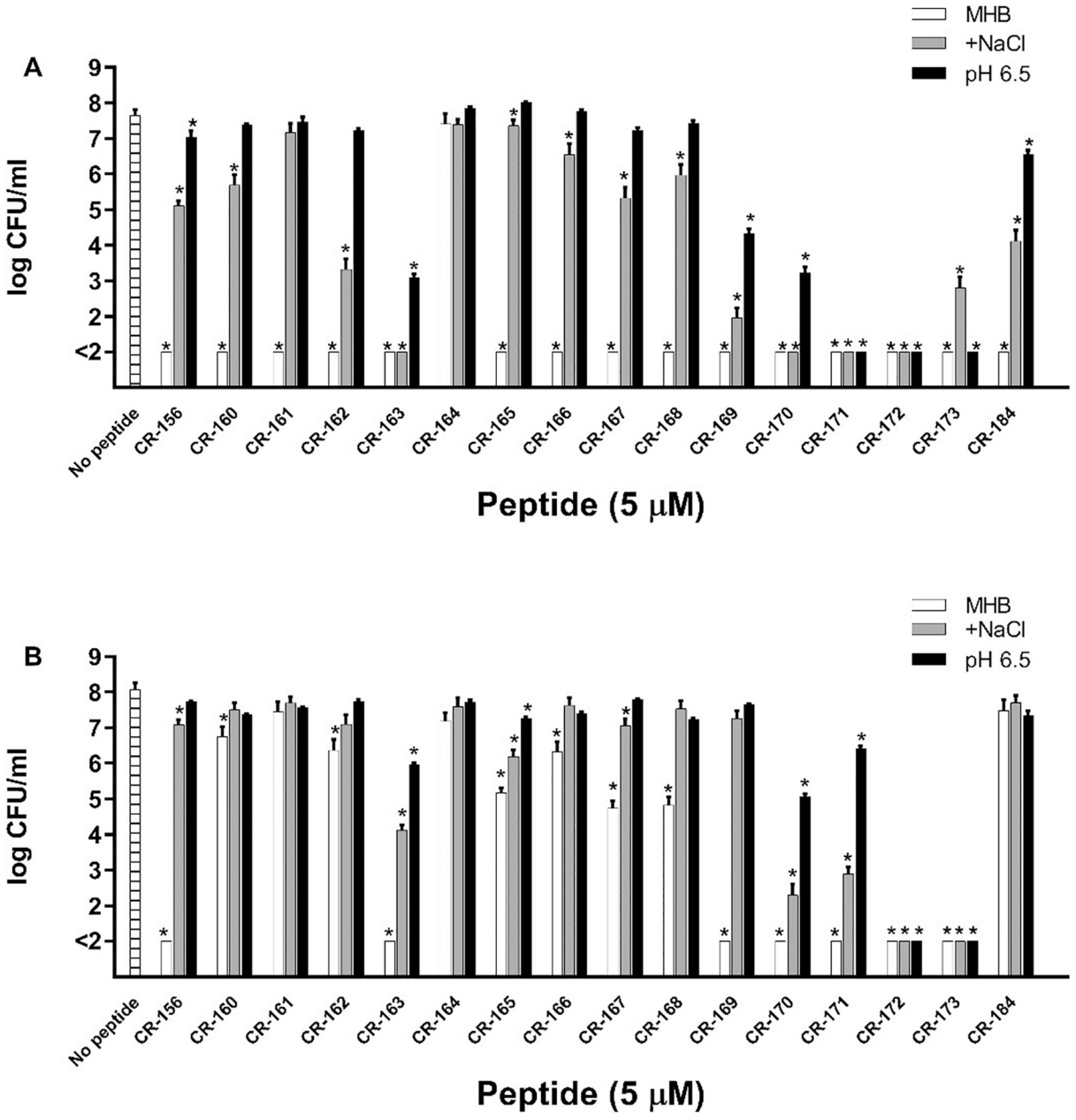

Fig. 1. Effects of saline and pH on the antibacterial activity of PepBiotics. Peptides (5 μM) were incubated with CF isolates of *P. aeruginosa* (A) or *S. aureus* (B) in MHB pH 7.4 (white), MHB pH 7.4 + 150 mM NaCl (grey) or MHB pH 6.5 (black bars) for 3 h after which viable bacteria were enumerated by plating out on agar plates. Shown are the averages +/− SEM from 2 to 4 independent experiments.* indicates $p < 0.05$

3.2. Antimicrobial activity screening of PepBiotics

Initial experiments indicated that, although CR-156 was active against clinical *P. aeruginosa* and *S. aureus* strains, its antimicrobial activity was sensitive to the presence of NaCl and low pH, as observed for other antimicrobial peptides [13,14]. Therefore, a larger set of alternative peptides, consisting of mutated and (mainly) extended derivatives of CR-156 was designed (Table 1) and tested for antimicrobial activity. Since intraspecies variability in peptide susceptibility was low (Table S1), a single *P. aeruginosa (*PA8*)* and a single *S. aureus* strain (SA21) was used in subsequent studies. All peptides were tested at a concentration of 5 μM in MHB in the presence or absence of 150 mM NaCl or in MHB with the pH adjusted to 6.5 resembling the lowered pH in the CF-lung [21]. It must be taken into account that the pH adjustment also increased ionic strength slightly (2.9 mM end concentration of HCl was added). Most of the peptides tested in standard MHB were active against *P. aeruginosa* reducing the viability of bacteria below the detection limit (Fig. 1A). However, addition of 150 mM NaCl reduced activity of most PepBiotics. For *S. aureus,* a comparable effect of NaCl on the activity of PepBiotics was observed (Fig. 1B). Similarly, a lowered pH to 6.5 generated a strong inhibitory effect on the antimicrobial activity against both *P. aeruginosa* and *S. aureus*. CR-172 and CR-173, however, retained full activity against both bacterial species under all medium conditions. CR-163, CR-170 and CR-171 decreased in activity but were still able to reduce bacterial viability by more than 99% compared to the (no peptide) control.

CR-163 was especially interesting since it only required 3 extra amino acids after the core sequence (which in itself is not antimicrobial at all, possibly due to its small size, unpublished results) to acquire high activity, while comparison of CR-163 with CR-162 show that a single amino acid difference can have a strong effect on activity.

3.3. Immunomodulation: LPS and LTA neutralizing activity

Many AMPs exhibit immunomodulatory activities, including neutralization of LPS-induced activation of immune cells. Various PepBiotics were tested for this immunomodulatory activity using RAW 264.7 macrophages stimulated for 24 h with 50 ng/ml *P. aeruginosa* LPS in the presence of 1, 5 or 20 μM PepBiotics. For several PepBiotics, dose-dependent neutralization of LPS was observed, including CR-163 which completely neutralized NO production to control levels at 20 μM (Fig. 2A). However, the most potent PepBiotics were CR-170 to CR-173,

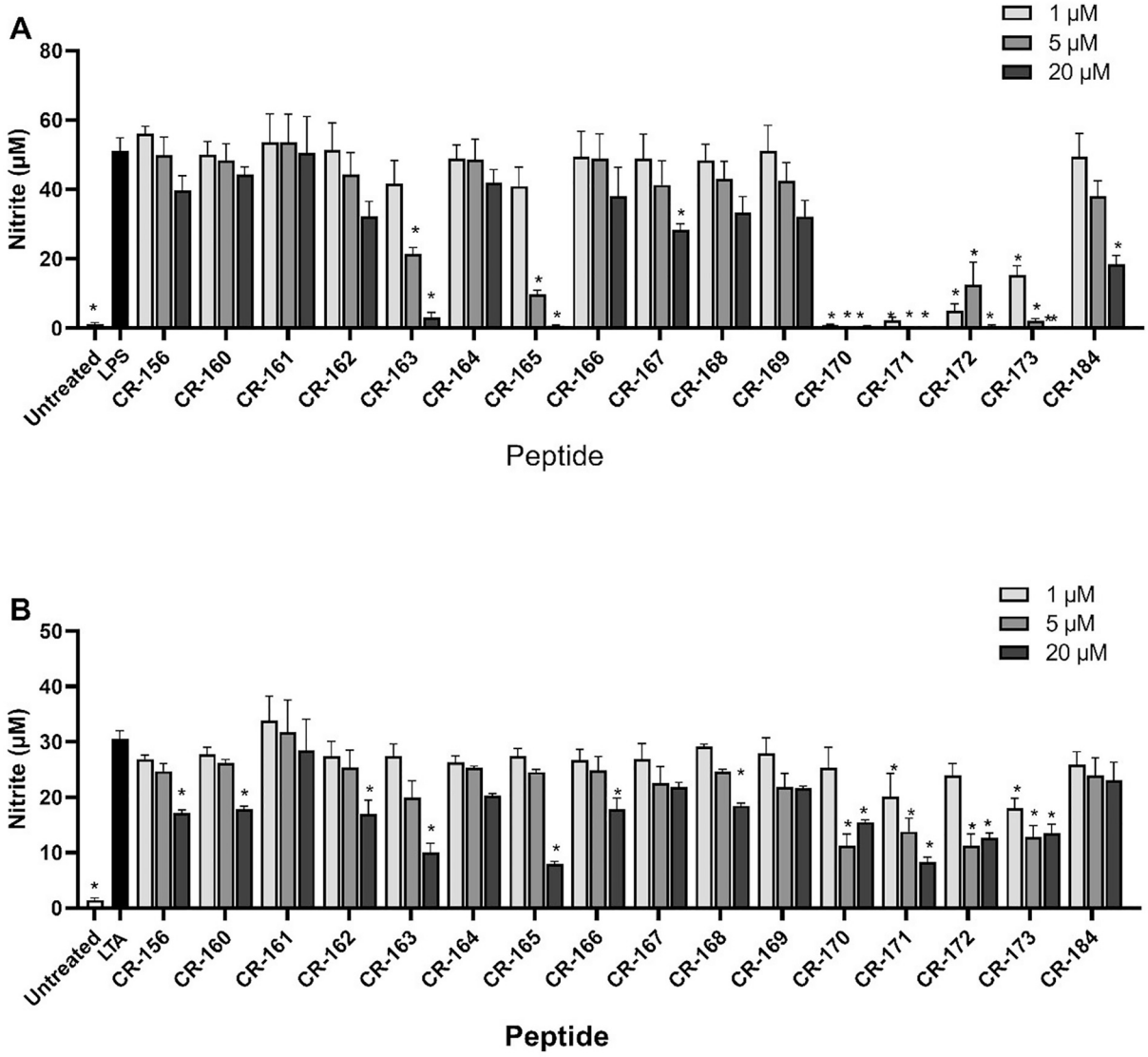

Fig. 2. Inhibition of LPS- and LTA-induced macrophage activation. RAW 264.7 cells were incubated with 50 ng/ml *P. aeruginosa* LPS (A) or 1 μg/ml *S. aureus* LTA (B) in the presence of 1, 5, or 20 μM PepBiotics. After 24 h NO production was measured. Shown are mean + SEM of 3 independent experiments performed in duplicate. * indicates $p < 0.05$, compared to LTA or LPS stimulation.

which completely neutralized LPS even at the lowest concentrations. Interestingly, this pattern of LPS neutralizing activity matches the antibacterial activity observed in Fig. 1, except for CR-165. This peptide neutralizes LPS significantly, yet has very low antibacterial activity against either *P. aeruginosa* or *S. aureus*. In a similar setup the inhibition of LTA-induced macrophage activation was determined by PepBiotics (Fig. 2B) and showed a similar pattern of inhibitory activity as for LPS neutralization. Several additional PepBiotics were able to significantly neutralize LTA compared to LPS, for example CR-163 and CR-165 at 20 μM. However, only partial inhibition of LTA was achieved for any peptide, while several PepBiotics were able to completely neutralize LPS to control, non-stimulated levels. A WST-1 assay was performed on RAW

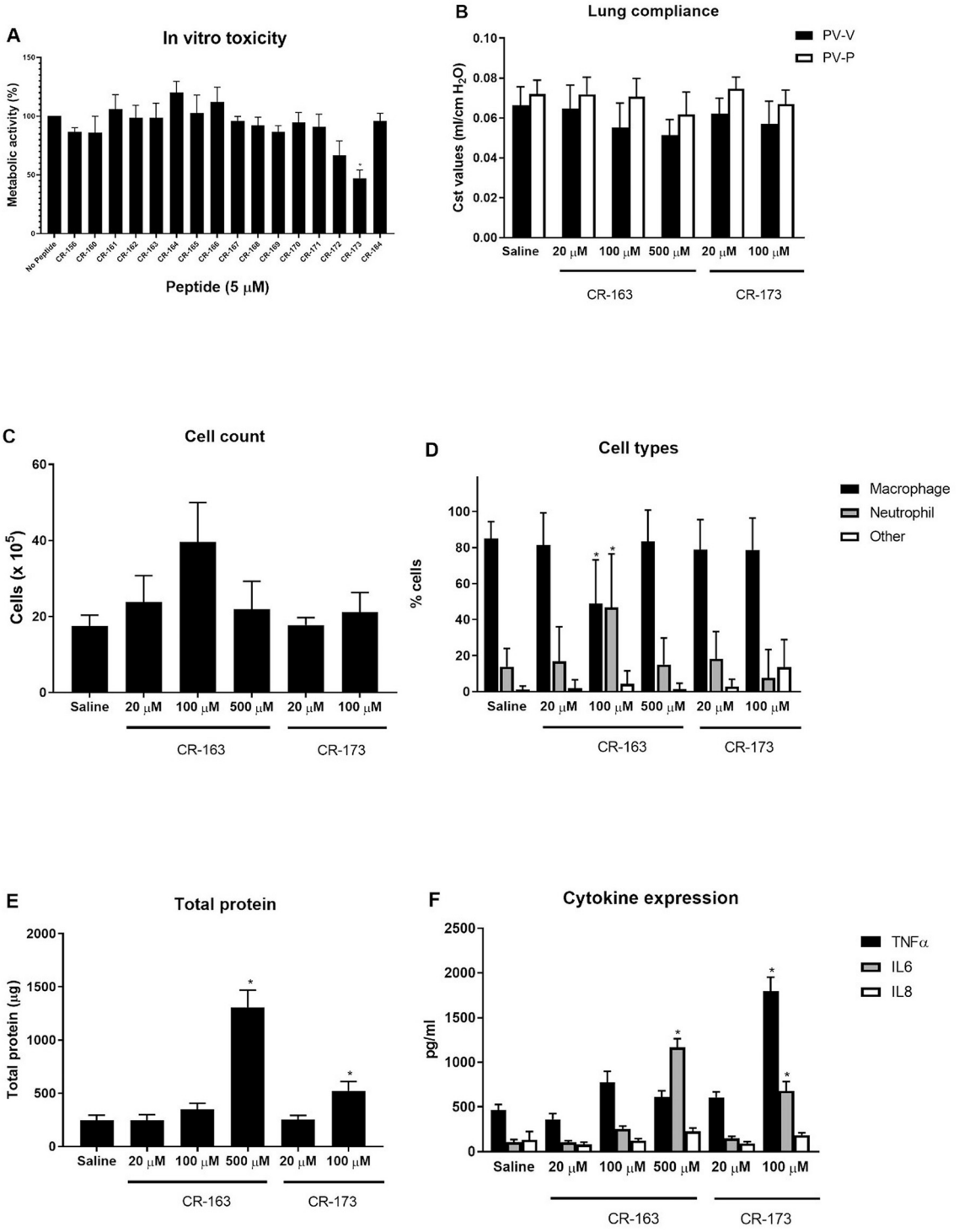

Fig. 3. Tolerance of PepBiotics. A) HNECs were incubated with 5 μM peptides for 3 h, after which WST was added for 30 min and metabolic activity was measured. Shown are mean + SEM of 4 independent experiment performed in triplicate. * indicates $p < 0.05$ compared to the'No Peptide' control. B-F) Mice were intra-tracheally injected with 50 μl of CR-163 (20, 100 and 500 μM) or CR-173 (20 and 100 μM). After 4 h, mice were euthanized and toxicity, lung function and injury parameters were measured. B) lung compliance, C) total cell count in the lung lavage, D) cell types in lung lavage, E) total protein levels in lung lavage, and F) cytokine concentration in lung lavage. Shown are mean + SEM of 6 or 7 mice per group. * indicates $p < 0.05$ compared to 'saline'.

264.7 cells incubated with 20 μM PepBiotics to determine if toxicity could account for the inhibition of LPS stimulation of some PepBiotics (Fig. S1). A small (non-significant) tendency for some peptides, (particularly CR-165 and CR-166) to decrease metabolic activity was observed. However, this result did not affect the conclusion about the LPS neutralizing capacity of PepBiotics.

Peptides without LPS or LTA did not activate murine macrophages (results not shown).

3.4. In vitro toxicity

The *in vitro* toxicity of PepBiotics was determined using primary human nasal epithelial cells (HNECs). These were incubated with 5 μM PepBiotics in BEPC for 3 h followed by measuring the cellular metabolic activity using WST-1 reagent. Under these conditions, most peptides did not affect the metabolic activity of HNECs and only incubation with CR-173 resulted in significantly lowered metabolic activity compared to the no-peptide control (Fig. 3A). CR-172 showed a tendency towards a lowered metabolic activity but this did not reach statistical significance.

3.5. In vivo toxicity

Two of the most active antibacterial and LPS-neutralizing peptides, but with strongest difference in their *in vitro* toxicity, CR-163 and CR-173, were tested for their toxicity *in vivo*, in order to obtain a clear indication about the correlation between *in vitro* and *in vivo* toxicity tests. Mice were injected intratracheally with 50 μl of either peptide at a concentration of 20, 100 or 500 μM. Mice were randomized over different groups. During the experiment, three out of the first 4 mice receiving the highest concentration of CR-173 had to be prematurely euthanized based on the health assessment score and no further animals were randomized in this experimental group due to toxicity. In the other groups the response to the peptide was assessed in several ways. At the end of the incubation period, mice were euthanized, and lung function was measured *ex vivo* (Fig. 3B). In addition, lung lavages were obtained to determine cytokine production, (immune) cell counts and total protein content as parameters for pulmonary toxicity and edema.

No significant differences were observed for any of the treatment groups with respect to animal weight (Fig. S2) and lung compliance (Fig. 3B). Analyses of the lung lavage showed that total cell counts were similar for all groups (Fig. 3C) but that the mice receiving 100 μM CR-163 had an altered ratio of macrophages and neutrophils compared to the other groups (Fig. 3D), including the mice receiving the higher (500 μM) CR-163 concentration. More obvious signs of mild lung injury were observed for total protein content, representative of starting edema (Fig. 3E) and the presence of cytokines (TNF-α and IL-6 but not IL-8) in the lung lavage (Fig. 3F), where a significant increase was seen for the 500 μM CR-163 and 100 μM CR-173 samples. Overall, the results indicate that CR-163 is well tolerated with only relatively small injurious effects at 500 μM, while CR-173's toxicity was much higher, correlating well with the *in vitro* toxicity data (Fig. 3A).

3.6. Development of antimicrobial resistance

Based on the toxicity and activity results, CR-163 and CR-172 were tested for developing antimicrobial resistance in *P. aeruginosa* and *S. aureus*. Bacteria were grown in the presence of sub-MIC concentrations of CR-163 and CR-172 with ciprofloxacin as a control with known susceptibility for development of resistance. As shown in Fig. 4, exposure of *P. aeruginosa* (panels A and C) and *S. aureus* (panels B and D) to ciprofloxacin led to a large steadily increase in MIC, while MICs for CR-163 remained nearly the same (Fig. 4, panels A and B). Similar results

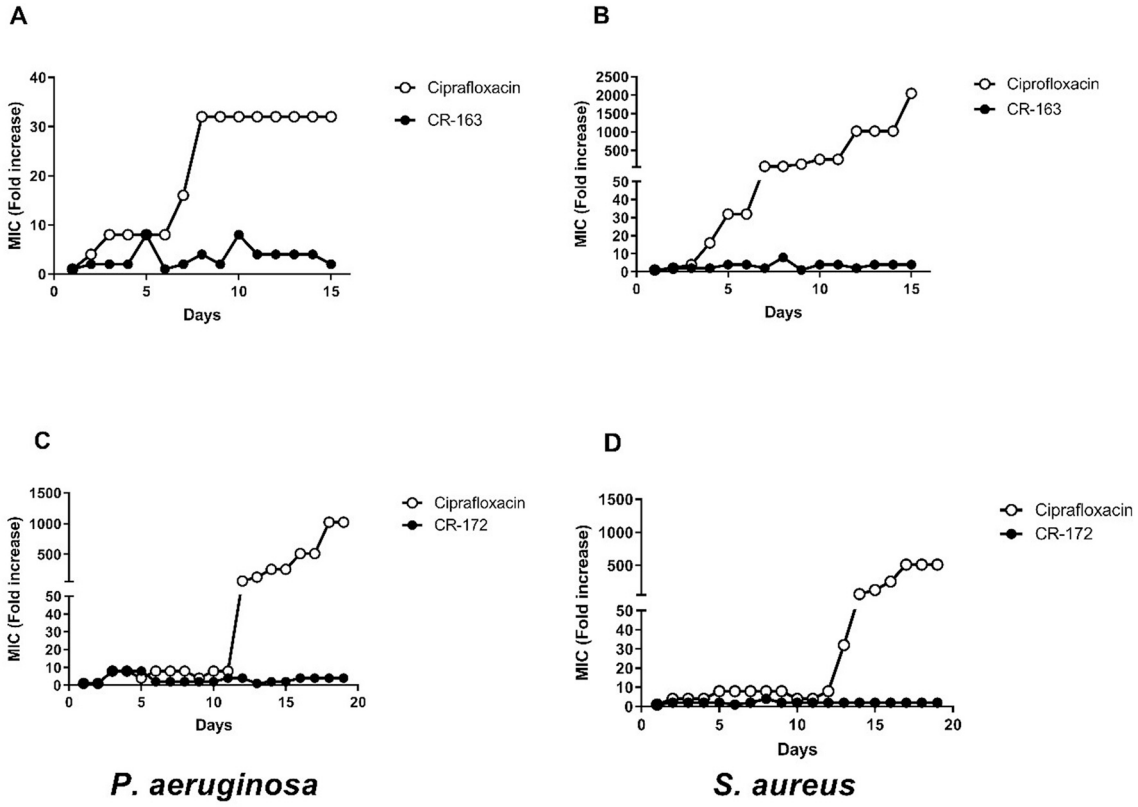

Fig. 4. CR-163 and CR-172 do not induce development of resistance in *P. aeruginosa* and *S. aureus*. Bacteria were grown in the presence of sub-MIC concentrations of ciprofloxacin and PepBiotics CR-163 and CR-172. Shown is the fold-increase in MIC of *P. aeruginosa* (panels A and C) or *S. aureus* (panels B and D) against CR-163 (panels A and B) or against CR-172 (panels C and D) as function of duration of sub-MIC exposure (days, X-axis). Included in all panels the curve obtained using ciprofloxacin (open symbols) instead of PepBiotics.

were observed for CR-172 (Fig. 4, panels C and D). The possibility of cross resistance was also tested. Ciprofloxacin-resistant strains and naïve strains were grown o/n in the absence of antibiotic and peptide, after which the MIC value against CR-163, CR-172 and ciprofloxacin was tested. These tests showed that ciprofloxacin resistance did not lead to co-resistance towards PepBiotics, while resistance towards ciprofloxacin was maintained despite the o/n growth in the absence of ciprofloxacin (Table 2).

3.7. Alanine scan

The CR-163 peptide, being the shortest core-sequence containing peptide with high antimicrobial activity and low toxicity, was used to perform an Ala replacement scan to determine which residues of the CR-163 sequence contributed to its antimicrobial activity. A panel of mutants was generated with each mutant having a single amino acid of the CR-163 sequence replaced by an Ala residue and their antimicrobial activity was tested against *P. aeruginosa* and *S. aureus*. As shown in Fig. 5, activity was decreased for most replacements and this was most pronounced when a Trp residue was replaced for an Ala, resulting in complete loss of activity. Substitution of charged residues only resulted in minor differences in activity. One substitution resulted in a remarkable result: replacement of Arg-1 by Ala completely abolished the anti-*P. aeruginosa* activity while activity of this Ala-1 mutant towards *S. aureus* was actually increased, indicating that a strong species-specific peptide was produced by this mutation.

3.8. Aromatic amino acid scan (Tryptophan scan)

The importance of Trp residues for antibacterial activity in the CR-163 sequence, as demonstrated by the Ala scan, was investigated in more detail by substitution of the Trp residues with other amino acids with bulky hydrophobic amino acids and testing of these mutants against *P. aeruginosa* and *S. aureus* (Fig. S3). Overall, most substitutions resulted in significantly decreased activity of the peptide as compared to the CR-163 peptide and multiple substitutions had a larger effect than single mutations, especially against *P. aeruginosa*.

3.9. Circular dichroism

The structure of CR-163 was determined using circular dichroism in the presence of membranes. For this, large unilamellar vesicles were composed of either POPG or POPC to which CR-163 was added. The zwitterionic PC vesicles did not induce a clear secondary structure in the short peptide as illustrated by the obtained spectrum characteristic for random coiled structures (Fig. 6). In contrast, interaction of CR-163 with POPG led to a clear change in conformation of the peptide with mainly α-helical characteristics, although exact calculations of secondary structures are generally not very accurate for small peptides such as CR-163. This mainly helical confiomation corresponds well with a secondary structure prediction of the peptide using structure prediction software PEP-FOLD3 [30](data not shown).

Table 2
Analysis of cross-resistance between PepBiotics and ciprofloxacin. *P. aeruginosa* and *S. aureus* were sub-cultured in the presence of ciprofloxacin until resistance emerged. These ciprofloxacin-resistant strains were subsequently analyzed for susceptibility towards CR-162 and CR-172 (and ciprofloxacin as control) followed by colony count assays. MIC values for naïve strains are included for comparison.

	P. aeruginosa		*S. aureus*	
	MIC Naïve	Cipro resistant	MIC Naïve	Cipro resistant
Cipro	<16 μg/ml	>1024 μg/ml	<16 μg /ml	>1024 μg/ml
CR-163	20 μM	20 μM	20 μM	20 μM
CR-172	10 μM	10 μM	20 μM	10 μM

3.10. NMR and ITC

To study the interaction of CR-163 with zwitterionic and negatively charged membranes in more detail, two additional techniques were used. The effect of CR-163 on POPG and POPC vesicles was investigated using ^{31}P solid state NMR (Fig. 6B). CR-163 caused a clear broadening of the ^{31}P powder pattern of POPG vesicles indicating that the peptides interact with the membrane. Note that the powder pattern solely results from the ^{31}P chemical shift anisotropy and is sensitive to both the headgroup mobility and orientation. CR-163 presumably stiffens the POPG headgroups, leading to a widening of the powder pattern. For POPC vesicles only a very minor effect was observed of the ^{31}P powder pattern. Increased signals intensity at the isotropic chemical shift was observed in the measurements, which indicates that CR-163 does neither cause strong curvature in the membranes nor the formation of spherical micelle-like structures. The interaction of CR-163 with negatively charged vesicles was confirmed using isothermal calorimetry (Fig. 6C). CR-163 added to POPG vesicles showed clear exothermic binding reflected by the observed production of heat in the thermogram. Upon saturation of binding this heat production was lost; CR-163 mixed with POPC vesicles resulted in very weak interaction (only visible at a 10-fold higher lipid concentration). Overall, the NMR and ITC results indicate that CR-163 interacts strongly with negatively charged membranes.

4. Discussion

Due to their high microbicidal activity with low or no induction of development of resistance, a major problem for therapeutic application of conventional antibiotics, AMPs are considered potential novel therapeutics to treat microbial infections. In our current study, new AMPs designated PepBiotics were specifically tested against CF-related pathogens in physiological conditions leading to CR-163 as the PepBiotic with the highest potential for future use in CF-related lung infections.

An important aspect of therapeutic development of novel (peptide-based) drugs is their toxicity towards host cells. There is a fine balance between antimicrobial activity and toxicity when optimizing peptide sequences, since both are (usually) based on interaction with membranes. Selectivity of AMPs for bacterial membranes is often attributed to the higher negative charge of the bacterial membrane and the presence of cholesterol in mammalian membranes. However, despite this selectivity, toxic concentrations of AMPs are generally in the same range as their MIC/MBC values, resulting in a very small therapeutic window [5]. For PepBiotics only minor cytotoxic effects were observed for most peptides, except for CR-173. Interestingly, CR-173 only has one amino acid (Pro-13) deleted compared to the less toxic CR-172. While both these peptides share similar MIC/MBC values (Fig. 1), this observation is in line with previous studies on the impact of prolines in mediating AMP selectivity. Prolines located at central positions in helical peptides, like in CR-172, often increase the selectivity of AMPs towards anionic membranes, probably due to generating a central hinge, that can alter the membrane binding and penetrating properties of AMPs [31–33]. In contrast, prolines positioned towards the N- or C-terminal of AMPs generally result in lower selectivity and activity. This is also illustrated by our studies showing a lower selectivity of CR-162 (proline near C-terminus) compared to proline-lacking CR-163, the latter having a lower MIC/MBC value while CR-162 and CR-163 show comparable *in vitro* toxicity.Toxicity was further examined *in vivo* using a mouse model (Fig. 3). Intratracheal delivery of 2 selected PepBiotics in various doses (comparable to concentrations used for inhaled antibiotics [34]) showed that up to (50 μl) of 100 μM CR-163 was well tolerated in the mouse lung while CR-173 showed more toxic effects, corresponding well with the *in vitro* toxicity assays However, i*n vivo* toxicity also depends on the delivery method used. Instead of instillation in the lung, an inhalation method could be envisioned for PepBiotics, or PepBiotics could be administered using a carrier such as pulmonary surfactant (see below). Such alternative application methods could have toxicity-lowering

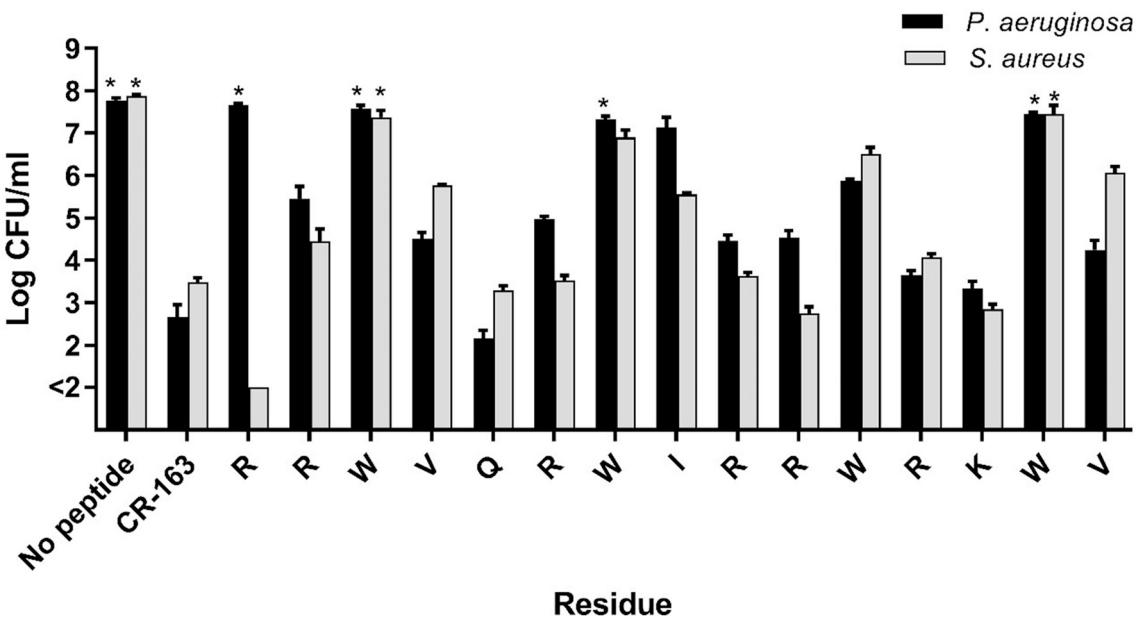

Fig. 5. Alanine scan of CR-163. The antimicrobial activity of 8 μM CR-163 and mutated forms, with each single amino acid substituted for Ala, was tested against *P. aeruginosa* (PA) and *S. aureus (SA)*. Peptides were incubated with bacteria for 3 h at 37 °C in MHB + 150 mM NaCl, after which viable bacteria were enumerated by plating out on agar plates. Shown are mean + SEM of 3 independent experiments. * $p < 0.05$ compared to the CR-163 peptide.

effects of PepBiotics potentially increasing the therapeutic window.

One very interesting aspect of naturally occurring AMPs is that they can express dual activity: in addition to direct antimicrobial killing properties they can also have a strong immunomodulatory, usually anti-inflammatory effect [35,36]. This dual activity to be able to kill a pathogen and subsequently reduce unwanted inflammation towards non-viable microorganisms, which we defined as 'silent killing' [37,38], is an important feature when future therapeutic use is envisioned for PepBiotics in a chronically inflamed CF lung. A first indication of immunomodulatory activity of PepBiotics was demonstrated here by their ability to neutralize LPS- and LTA-induced activation of macrophages (Fig. 2). Although not specifically tested, it is likely that direct binding of peptide to LPS occurs, thereby inhibiting TLR4 interaction, as is observed for other AMPs [39]. Although molecular characteristics of AMPs such as hydrophobicity and positive charge are likely involved in LPS binding, not all AMPs actually bind (and neutralize) LPS [2,40].

Based on its strong antimicrobial activity in high ionic strength and lowered pH, low toxicity, immunomodulatory activity and relatively short length, the 15 amino acid peptide CR-163 was chosen for more in depth structural studies. An alanine screen revealed that hydrophobic residues in the peptide sequence, especially Trp residues, were elemental for its antimicrobial activity (Fig. 5 and Fig. S3). This preference for Trp in AMP sequences is quite common in AMPs since Trp residues preferentially interact with the interfacial region of membranes [41].

Although PepBiotics were tested and proven active under physiological conditions (this study), several aspects have to be taken in consideration with regard to the use of these peptides to treat pulmonary bacterial infections in CF patients. One major difference between our *in vitro* studies and the *in vivo* infection is that in CF most pulmonary pathogens will be present in biofilm-like structures. Therefore, the anti-biofilm activity of CR-163 and other peptides should be investigated. In terms of drug delivery, intravenous application of peptides is not the preferred route because of the low stability of AMPs in blood, and the large dilution of the compound before it reaches the lung. Therefore direct pulmonary delivery of drugs, or delivery via a carrier like pulmonary surfactant would be preferred since this has the advantage of achieving rapid and high bioavailability while limiting side effects [34,42–44].

For further therapeutic development it is recommended to investigate the activity of PepBiotics against other CF-related pathogens. Although *P. aeruginosa* and *S. aureus* are the most commonly encountered bacterial species in CF, usually more complex highly heterogeneous microbial infections are present that also include fungal species (e.g. *Aspergillus fumigatus*) [45]. Recently we reported that several PepBiotics exhibit strong antifungal properties [46], demonstrating its broader therapeutic potential. Fungicidal effects were shown against a broad panel of clinically important fungal species, including azole-resistant *Aspergillus fumigatus*, a fungal infection often associated with CF, that leads to allergic bronchopulmonary aspergillosis and clearly associated with airway damage and lung function decline. It is envisioned that combination therapy by using mixtures of carefully selected peptides might have superior synergistic effects in cases where respiratory co-infection has established. We aim to further investigate and develop additional designs of bacterial species-specific PepBiotics and study the synergistic potential of combinations of PepBiotics. Importantly, PepBiotics will be investigated for its potential to enhance the efficacy of simultaneously delivered conventional antibiotics by combining different mechanisms of action.

In conclusion, this study characterizes PepBiotics, a panel of novel peptide-based antimicrobial drugs that exhibit strong and robust antimicrobial activity against CF-pathogens, with tolerable toxicity levels, low resistance-development and LPS-neutralizing properties. Ultimately, we aim to develop PepBiotics as a novel anti-infective that can be applied into the airways of patients via nasal or pulmonary aerosolic administration by the use of conventional inhalation devices. Successful treatment of CF-patients provides an opportunity to develop PepBiotics for other respiratory infectious diseases caused by bacterial or fungal pathogens.

Author contribution

MvE, AvD, EB, NvO, RA, SvdW, RLG, MK, SJ, RAWV: investigation and methodology.

MvE, AvD, EJAV and HPH project administration and conceptualization. CvdE, HGMA, MW, MH and HPH supervision. EJAV, MvE, RAWV and HPH: writing-review & editing.

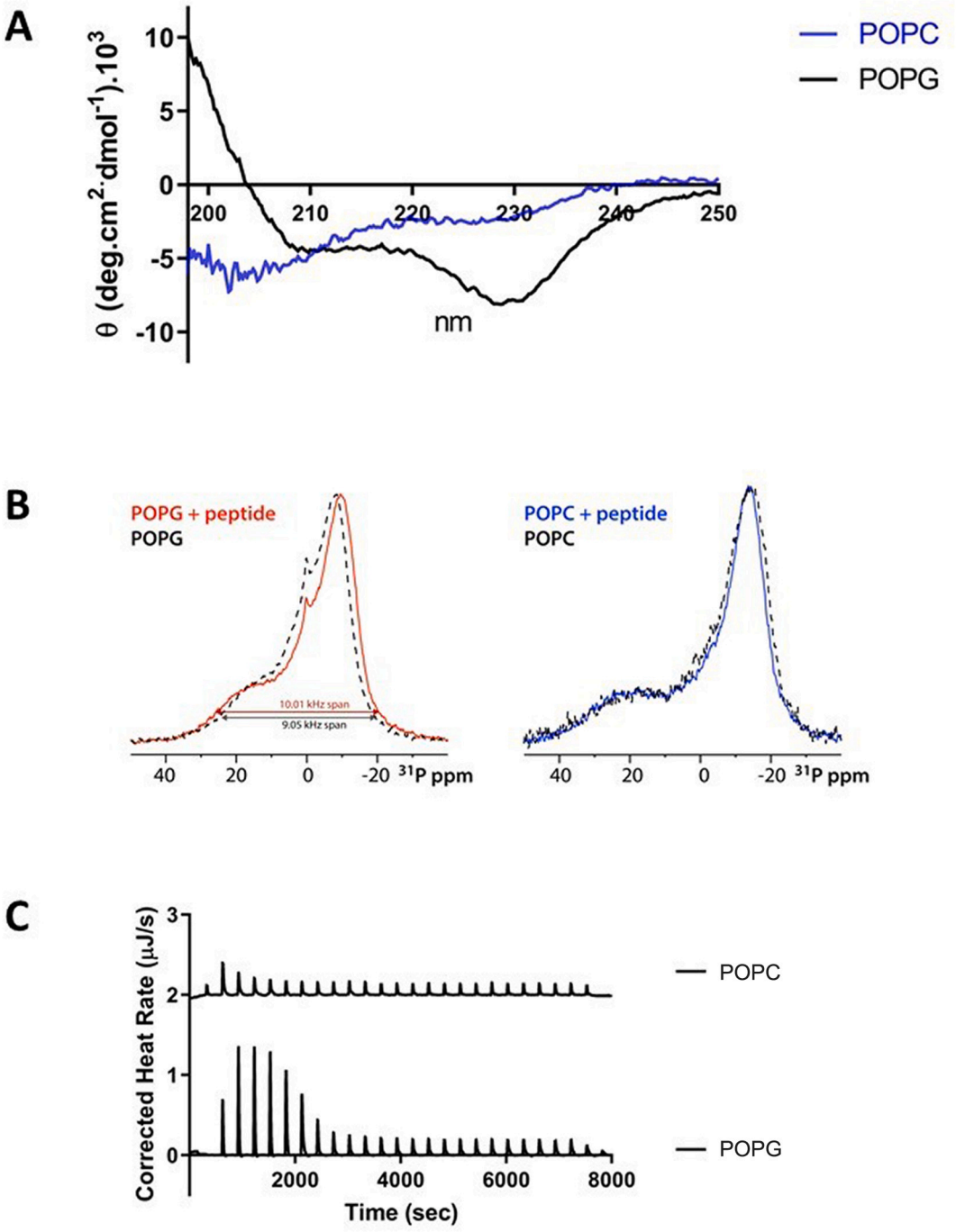

Fig. 6. Interaction of CR-163 with membranes. A) CR-163 was mixed with phospholipids at a 1:50 ratio and Circular Dichroism spectra were recorded from 250 to 190 nm. Shown is the average of 4 recordings. B) CR-163 was mixed with POPG or POPC vesicles (1:50, mol:mol) in 5 mM Hepes, pH 7.5, 50 mM NaCl. Static ^{31}P solid-state NMR spectra were acquired at 500 MHz magnetic field (^{1}H-frequency). C) Isothermal titration calorimetry: shown are representative thermographs of 2 µl injections of 320 µM CR-163 into a 169 µl solution containing 0.15 mg/ml POPG or 1.5 mg/ml POPC unilamellar vesicles.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbagen.2021.129951.

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