# Differential In Vitro and In Vivo Toxicities of Antimicrobial Peptide Prodrugs for Potential Use in Cystic Fibrosis.

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# Differential *In Vitro* and *In Vivo* Toxicities of Antimicrobial Peptide Prodrugs for Potential Use in Cystic Fibrosis

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**There has been considerable interest in the use of antimicrobial peptides (AMPs) as antimicrobial agents for the treatment of many conditions, including cystic fibrosis (CF). The challenging conditions of the CF patient lung require robust AMPs that are active in an environment of high proteolytic activity but that also have low cytotoxicity and immunogenicity. Previously, we developed prodrugs of AMPs that limited the cytotoxic effects of AMP treatment by rendering the antimicrobial activity dependent on the host enzyme neutrophil elastase (NE). However, cytotoxicity remained an issue. Here, we describe the further optimization of the AMP prodrug (pro-AMP) model for CF to produce pro-WMR, a peptide with greatly reduced cytotoxicity (50% inhibitory concentration against CFBE41o- cells, >300 μM) compared to that of the previous group of pro-AMPs. The bactericidal activity of pro-WMR was increased in NE-rich bronchoalveolar lavage (BAL) fluid from CF patients (range, 8.4% ± 6.9% alone to 91.5% ± 5.8% with BAL fluid; $P = 0.0004$), an activity differential greater than that of previous pro-AMPs. In a murine model of lung delivery, the pro-AMP modification reduced host toxicity, with pro-WMR being less toxic than the active peptide. Previously, host toxicity issues have hampered the clinical application of AMPs. However, the development of application-specific AMPs with modifications that minimize toxicity similar to those described here can significantly advance their potential use in patients. The combination of this prodrug strategy with a highly active AMP has the potential to produce new therapeutics for the challenging conditions of the CF patient lung.**

Cystic fibrosis (CF) is an autosomal recessive disorder caused by mutations in the gene coding for the cystic fibrosis transmembrane conductance regulator (CFTR) (1). In the respiratory tract, CFTR dysfunction leads to a dehydrated and volume-depleted airway surface liquid (ASL) (2, 3). The dysfunction critically impairs the host defensive response to infection, increasing the contact time between bacteria and the epithelium, and leads to severe illnesses and progressive pulmonary damage (2, 4, 5). *Pseudomonas aeruginosa* is the most important pathogen in patients with CF (4, 6, 7). The resulting chronic infection, localized to the endobronchial space, is difficult to remove (8) and is the primary cause of morbidity and mortality (9).

The neutrophil-dominated immune response releases large quantities of the serine protease neutrophil elastase (NE) into the endobronchial space, contributing to airway inflammation, mucus hypersecretion, and tissue damage (10–12). The nonresolving inflammatory response leads to long-term reductions in lung function and is associated with premature death (7, 8, 13). Neutrophils represent approximately 70% of the airway inflammatory cell population in patients with CF but only 1% of the airway inflammatory cell population in healthy individuals (14). There are various reasons for this, and these are mostly related to elevated neutrophil chemokine levels in the CF patient lung, mainly due to the ineffective clearance of *P. aeruginosa* (10). High NE levels overwhelm epithelial antiprotease defenses and can inactivate other components of the immune response, such as complement and immunoglobulins (14). Aggressive antibiotic therapy with drugs, such as inhaled tobramycin, is recommended, but their efficacy as anti-infective therapy is limited (6).

One potential source of new anti-infectives for CF is antimicrobial peptides (AMPs) (15, 16). These short amphipathic peptides, composed of hydrophobic and charged amino acids, are crucial components of the innate immune system. Their antimicrobial activity exploits a fundamental charge difference between bacterial and mammalian cell surfaces, and they have multiple mechanisms of killing (17). Many AMPs are also immunomodulatory, being capable of recruiting and stimulating other components of innate immunity (18). These properties minimize the propensity for bacteria to develop resistance during or after therapy, making AMPs attractive anti-infectives for patients with CF who are infected with antibiotic-resistant microorganisms (19).

Another rationale for the use of AMPs as exogenous therapeutics in patients with CF is to compensate for the cleavage and inactivation by pulmonary proteases of endogenous AMPs, such as LL-37 (10) and the β-defensins (20). Some AMPs may also be

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inactivated by the potentially decreased pH of airway surfaces in patients with CF (21).

At high concentrations, many AMPs are active against both eukaryotic and bacterial membranes. This potential host toxicity has hampered the development of AMPs as antimicrobial agents. Different approaches have been explored to exploit the antimicrobial activity of AMPs while limiting their cytotoxic effects, including a prodrug strategy (22). This involves the attachment of prodrug moieties that reversibly inactivate the AMP until desirable conditions are met. This approach was recently applied to the normally highly toxic AMP melittin and was found to reduce its *in vitro* cytotoxicity and allow its use in a mouse model of cancer to reduce the tumor size (23).

We have previously designed NE-sensitive AMP prodrugs (pro-AMPs) by adding an oligoglutamic acid pro-moiety to reduce the net charge (which lowered the antimicrobial activity and cytotoxicity) and an NE-cleavable linker, AAAG, for activation in the CF patient lung. The colocalization of *P. aeruginosa* and NE allows the potential cytotoxic effects of the AMPs (pro-HB43 and pro-P18) to be confined to the site of infection (16). However, a significant scope to improve the selectivity of pro-AMPs remained. We therefore investigated the redesign of the pro-AMPs for use in the treatment of *P. aeruginosa* infection in CF patients with a view to limiting the cytotoxic and immunogenic impacts of the peptides. To achieve this, new sources of active AMP were explored.

One such AMP is WMR, a peptide developed from innate immunity peptides found in the hagfish *Myxine glutinosa*, where activity in an environment of high salinity is required. It demonstrated high levels of activity against *P. aeruginosa* and low levels of cytotoxicity against human cells (24–26). Another AMP chosen for prodrug modification, WR12, comes from a series of salt-resistant peptides synthesized by Deslouches et al. (27, 28). Its activity and cytotoxicity characteristics are also promising in the CF context, as it was shown to have good activity against a panel of 100 *Pseudomonas* isolates from CF patients (27, 28).

In this study, using WMR, we identified an AMP prodrug candidate for use in patients with CF with improved salt resistance and bactericidal activity in CF patient bronchoalveolar lavage (BAL) fluid as well as lower cytotoxicity and immunogenicity. The cytotoxicity of the new pro-AMP was also investigated *in vivo* and compared with that of the previous best pro-AMP candidate, pro-P18. The new pro-AMP demonstrates the characteristics required of an effective anti-infective in CF patients. In addition, the necessity for protease-resistant D-amino acids in the design of the pro-AMPs and their effect on host neutrophils were investigated. The results demonstrate how a very low level of cytotoxicity and targeted antibacterial activity are achievable with AMPs in patients with CF, supporting their further evaluation and development as new therapeutic agents for use by patients with this disease.

## MATERIALS AND METHODS

**Strains and clinical isolates.** The laboratory strain PAO1 (ATCC 15692; American Type Culture Collection, Manassas, VA, USA) was used as a reference. *P. aeruginosa* clinical isolates from CF patients were obtained from the diagnostic microbiology laboratory of Beaumont Hospital, Dublin, Ireland, a seven-bed tertiary referral center with a large population of adult CF patients. Isolate identity was confirmed by the BBL DrySlide oxidase test (BD, USA), the C-390 Diatab disk test (Rosco Diagnostics, Germany), and matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS; Bruker, Germany).

**CF BAL fluid collection.** Samples of CF patient BAL fluid (CF BAL fluid) were collected from consenting CF patients. Non-CF BAL fluid was collected from patients with stage I or II sarcoidosis from a previous study. Both protocols for collection were approved by the Beaumont Hospital Research (Ethics) Committee. The NE content was determined by measuring the cleavage of *N*-methoxysuccinyl-Ala-Ala-Pro-Val-*p*-nitroanilide as described previously (16).

**Blood collection.** Blood samples were collected from consenting healthy individuals. The protocol was approved by the Royal College of Surgeons in Ireland (RCSI) Research Ethics Committee. Blood was collected in 7.5-ml tubes containing heparin lithium-coated beads (Sarstedt, Ireland).

**Peptide synthesis.** Prodrugs were synthesized on the basis of the sequences of four active peptide amides: D-HB43 (fakllaklakkll), D-P18$^{8\ Leu}$ (kwklfkklpkflhlakkf), D-WMR$^{3,6\ Leu}$ (wglrrllkygkrs), and D-WR12 (rwwrwwrrwwrr)(lowercase indicates D-amino acids). The isoleucine residues normally found in P18 and WMR were replaced by leucine. This has previously been shown not to negatively impact the activity of P18, while it decreases the cost associated with the use of D-isoleucine in synthesis (29). This modification has not previously been made to WMR and was investigated by comparison of the activity of D-WMR to that of L-WMR (WGIRRILKYGKRS). Here, the D-AMPs are referred to as HB43, P18, WMR, and WR12.

The parent sequences were assembled by automated solid-phase peptide synthesis on a 433A synthesizer (Applied Biosystems, United Kingdom) from 9-fluorenylmethoxy carbonyl-protected D-amino acids (Merck Chemical, United Kingdom) with HATU [*O*-(7-azabenzotriazole-1-yl)- *N*,*N*,*N*,*N*′-tetramethyluronium hexafluorophosphate; ChemPep, USA]/DIEA (*N*,*N*-diisopropylethylamine; Sigma-Aldrich, Ireland) coupling chemistry from a Rink amide MBHA (4-methylbenzhydrlamine) resin (Merck Chemical, United Kingdom). For the pro-AMPs, elongation with the AAAG linker and glutamic acids and N-terminal acetylation were carried out manually with L-amino acids.

Chromatographic analysis and purification were performed on a Galaxie high-pressure liquid chromatography (HPLC) system (Varian, USA) and a BioCAD Sprint perfusion chromatography workstation (PerSeptive Biosystems, United Kingdom), respectively, using Gemini (particle size, 5 μm; $C_{18}$; 110 Å) columns (Phenomenex, United Kingdom). Purified peptides were finally characterized by analytical HPLC and MALDI-TOF MS using the α-cyano-4-hydroxy-cinnamic acid matrix.

**Enzymatic cleavage of pro-AMPs.** Each pro-AMP was incubated with 5 or 20 μg/ml purified NE (Elastin Products Company, USA) at 37°C in phosphate-buffered saline (PBS), pH 7.4. Samples were removed from the incubation mixture and analyzed by HPLC and MALDI-TOF MS. Before the analysis of pro-AMPs cleaved by CF BAL fluid, samples were first diluted in an equal volume of distilled $H_2O$ ($dH_2O$) and filtered using a 10-kDa Centrisart I ultrafiltration system (Sartorius, Ireland), following the manufacturer's instructions.

**Susceptibility testing.** MICs were determined using the broth microdilution method according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (30) with the modifications for cationic peptides described by Wu and Hancock (31). Briefly, serial doubling dilutions of peptide were made in a sterile solution containing 0.2% (wt/vol) bovine serum albumin (BSA) and 0.01% (vol/vol) acetic acid. These were added to a 96-well microtiter plate with a $1.5 \times 10^5$-CFU/ml inoculum of *P. aeruginosa* reference strain PAO1 or clinical isolates in Mueller-Hinton (MH) broth (non-cation adjusted; Oxoid, United Kingdom). The lowest peptide concentration showing no visible growth was recorded as the MIC.

**Bactericidal killing activity.** *P. aeruginosa* strain PAO1 and the clinical isolates were grown overnight at 37°C on MH agar. Isolated colonies were used to prepare suspensions to the density of a 1.0 McFarland standard using a Densichek meter (bioMérieux, Ireland), and the suspensions were further diluted 1/100 in potassium phosphate buffer, pH 7.4, containing 0.2% BSA. Assays were carried out in microcentrifuge tubes with

**TABLE 1** MIC values of parent AMPs, cleaved AMPs, and pro-AMPs for *P. aeruginosa* PAO1 and CF clinical isolates PABH01 to PABH04

| Peptide | Sequence[a] | MIC (μg/ml) for *P. aeruginosa* strain: | | | | |
|---|---|---|---|---|---|---|
| | | PAO1 | PABH01 | PABH02 | PABH03 | PABH04 |
| WMR | wglrrllkygkrs-$NH_2$ | 64 | 16 | 16 | 32 | 1 |
| L-WMR | WGIRRILKYGKRS-$NH_2$ | 64 | 32 | 64 | 32 | 2 |
| AAG-WMR | AAGwglrrllkygkrs-$NH_2$ | 32 | 8 | 16 | 32 | 16 |
| Pro-WMR | Ac-EEEEAAAGwglrrllkygkrs-$NH_2$ | >64 | >64 | >64 | >64 | >64 |
| L-Pro-WMR | Ac-EEEEAAAGWGLRRLLKYGKRS-$NH_2$ | >64 | >64 | >64 | >64 | >64 |
| AAG-WR12 | AAGrwwrwwrrwwrr-$NH_2$ | 32 | 8 | 32 | 16 | 32 |
| Pro-WR12 | Ac-EEEEAAAGrwwrwwrrwwrr-$NH_2$ | >64 | >64 | >64 | >64 | >64 |

[a] Amino acids in uppercase letters are L-amino acids, and those in lowercase letters are D-amino acids.

peptide solution, a 10% (vol/vol) *P. aeruginosa* suspension (approximately $1.5 \times 10^6$ CFU/ml), and 10 mM potassium phosphate buffer, pH 7.4, containing 0.2% BSA. The assay mixtures were incubated in a shaking incubator at 37°C and 200 rpm for 1 h and then diluted 1/10 with 0.95% (wt/vol) NaCl. A 100-μl aliquot was spread onto MH agar and incubated overnight at 37°C. Killing activity (in percent) was calculated from viable counts as the number of CFU per milliliter from assays containing peptides compared to the number of CFU per milliliter from control assays not containing the peptide. The effects of purified NE (20 μg/ml), CF BAL fluid (25%, vol/vol), and NaCl (50 to 250 mM) on killing activity were determined by the addition of these to the assay mixtures and the inclusion of appropriate controls. Statistical analyses of the data were carried out using GraphPad Prism software and the two-tailed unpaired *t* test.

**Cell culture.** The human F508del homozygous CFBE41o- bronchial and CFTE29o- tracheal epithelial cell lines were a gift from D. Gruenert (California Pacific Medical Centre Research Institute, San Francisco, CA) (32, 33). Cells were cultured in minimal essential medium (MEM) supplemented with 10% (vol/vol) fetal calf serum (FCS), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere with 5% $CO_2$.

**Neutrophil isolation.** Blood was drawn in 7.5-ml heparinized S-Monovette tubes (10 U/ml of heparin; Sarstedt, Germany). Neutrophils were purified by dextran sedimentation and Lymphoprep centrifugation (34). In brief, a 4-ml aliquot of 10% (wt/vol) dextran ($M_r$, 500,000; Sigma, Ireland) was added to 40 ml of freshly collected blood; these components were gently mixed and allowed to settle. Five milliliters of Lymphoprep density gradient medium (Axis-Shield, United Kingdom) was underlaid under 15 ml of the resulting upper layer containing leukocytes, and the combination was centrifuged at 800 × *g* for 10 min (Heraeus Megafuge 1.0 centrifuge; Kendro Laboratory Products, Germany). The remaining erythrocytes were lysed by a brief hypotonic shock by the addition of 25 ml of $dH_2O$, followed by the immediate addition of an equal volume of 1.8% (wt/vol) NaCl. After a further centrifugation at 500 × *g*, the neutrophil-rich pellet was resuspended in 1 ml PBS containing 5 mM glucose. Neutrophils were quantified using a hemocytometer, and viability was assessed by trypan blue exclusion assays (VWR, Ireland). The results confirmed that the viability of the freshly purified neutrophils was above 98%.

**Cytotoxicity assays.** CFBE41o- and CFTE29o- cells were seeded on 96-well plates at a density of $3 \times 10^4$ cells/well, and neutrophils were suspended in microcentrifuge tubes at a density of $5 \times 10^5$ cells/tube. The epithelial cells were incubated for 24 h at 37°C. The cells were then treated in triplicate with 0.2 to 300 μM of the peptides and their prodrugs in serum-free medium, consisting of MEM for the epithelial cells and RPMI 1640 medium for neutrophils. Incubation was for 24 h for the epithelial cells and 3 h for the neutrophils. After incubation, the growth medium was removed and the cells were incubated with 500 μg/ml of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT; Sigma, Ireland) in serum-free MEM or RPMI 1640 medium. The incubation time was 4 h for epithelial cells and 2 h for neutrophils. The MTT solution was removed and replaced with 100 μl of dimethyl sulfoxide, the components were mixed by gentle shaking, and the absorbance at 560 nm was recorded. The 50% inhibitory concentrations ($IC_{50}$s), defined as the peptide concentrations that resulted in 50% cell death, were calculated using GraphPad Prism software from the resulting sigmoidal dose-response curve.

**Cytokine release assay.** CFBE41o- cells were seeded on 24-well plates at a density of $1.5 \times 10^5$ cells/well and incubated for 24 h at 37°C. The cells were treated in triplicate with sub-$IC_{50}$s of the peptides and their prodrugs in MEM containing 1% FCS for 24 h. The plates were centrifuged at 700 × *g* for 5 min, and the cell supernatant was removed. The cytokine concentration of each supernatant was measured using a human proinflammatory panel (V-Plex Plus kit; MSD, Ireland) according to the manufacturer's instructions. Lipopolysaccharide (LPS) at a concentration of 50 μg/ml was used as a positive control.

***In vivo* toxicity studies.** All *in vivo* studies were granted ethical approval by the Regierungspräsidium (Regional Council) of Karlsruhe, Baden-Württemberg, Germany (reference G-284/14). C57BL/6 mice (11 to 15 weeks old) were sedated using 3% (vol/vol) isoflurane in $O_2$ at a flow rate of 2 liters/min. They were treated intratracheally with 50 μl of a 1-mg/ml peptide solution in PBS and again 6 h later. Mice that died were not processed further. At 24 h after the first dose, the mice were deeply anesthetized by intraperitoneal injection of ketamine-xylazine (120 mg/kg of body weight and 16 mg/kg, respectively), and then a lung lavage with cold PBS was carried out as described previously (35). Analysis of the BAL fluid cell pellet was undertaken microscopically, using trypan blue dye exclusion for determination of the total cell count and May-Grünwald-Giemsa staining for determination of the relative proportion of each cell type. Analysis of the levels of the cytokines keratinocyte chemoattractant (KC) and tumor necrosis factor alpha (TNF-α) in the cell-free supernatant was carried out using a V-Plex Plus proinflammatory panel 1 (mouse) kit (MSD, Ireland) according to the manufacturer's instructions. Statistical analyses of the data were carried out using GraphPad Prism software and the two-tailed unpaired *t* test.

## RESULTS

**Pro-WMR and pro-WR12 are cleaved by NE.** Both pro-WR12 and pro-WMR were synthesized. Two enantiomeric peptides (assembled from L- or D-amino acids) of WMR were prepared with a modification in the D-peptide where isoleucine residues were replaced by leucine, a substitution previously described (16). To ensure that the modification was not deleterious to activity, the MICs of L-WMR and the modified D-WMR were compared and showed that the latter was more active against three of the four clinical isolates of *P. aeruginosa* (Table 1). Hereafter, D-WMR is referred to as WMR. The activity of both WR12 and WMR was maintained in the presence of high concentrations of NaCl and was reduced only slightly in the presence of 250 mM NaCl (see Fig. S1 in the supplemental material). Both propeptides of WR12 and WMR were cleaved by NE, with alanine and glycine remaining;

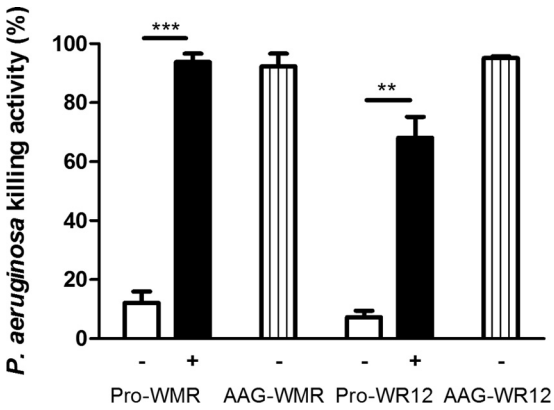


**FIG 1** Effect of 20 μg/ml NE on the bactericidal activity of pro-AMPs against *P. aeruginosa* PAO1. NE was added to assay mixtures containing 3.125 μg/ml pro-AMPs. The killing activities shown are the means ± SEMs from three independent assays carried out in duplicate. NE alone had no killing activity (data not shown). Statistical analyses were carried out using an unpaired two-tailed *t* test. **, $P < 0.01$; ***, $P < 0.001$.

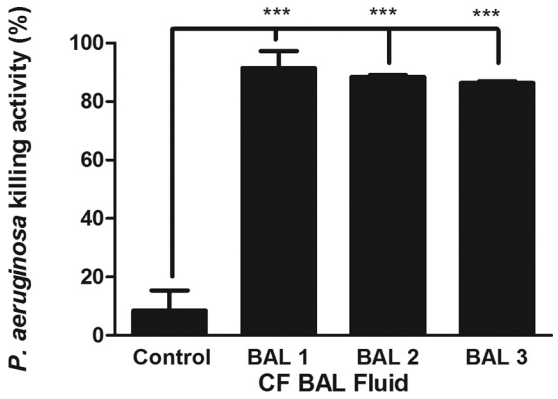


**FIG 2** Effect of 25% (vol/vol) CF BAL fluid on the bactericidal activity of pro-WMR (25 μg/ml) against *P. aeruginosa* PAO1 in the presence of 300 mM NaCl (the control was NaCl alone). The values shown are the means ± SEMs from three independent assays with three different BAL fluid samples (BAL 1 to BAL 3), where the activity due to CF BAL fluid alone was subtracted. Statistical analyses were carried out using an unpaired two-tailed *t* test. ***, $P < 0.001$.

the main cleavage products were AG/AAG-AMP. Both AAG-WR12 and AAG-WMR were therefore synthesized as controls. The bactericidal activity of both pro-AMPs increased in the presence of 20 μg/ml NE (Fig. 1). For example, for 3.125 μg/ml of pro-WMR, the bactericidal activity increased from 12.1% ± 3.9% to 93.8% ± 2.8% ($P < 0.0001$). Consistent with these results, the MICs were also higher for the pro-AMPs than the cleaved products (Table 1). The additional residues present on each cleavage product, i.e., alanine and glycine, did not affect the MICs for most isolates (Table 1).

**Pro-WMR is active in CF BAL fluid.** Difficulty with synthesis and poor yields precluded the further investigation of pro-WR12. Incubation of pro-WMR with CF BAL fluid and analysis by HPLC/MALDI-TOF MS resulted in the same cleavage products obtained with purified NE, with the D-amino acid active sequence being unaffected. The bactericidal activity of pro-WMR in BAL fluid was investigated at a higher concentration of 25 μg/ml with 300 mM NaCl, which was previously used to overcome antagonism from BAL fluid components by reducing nonspecific electrostatic interactions (16). Incubation with 25% (vol/vol) CF BAL fluid and 300 mM NaCl increased the bactericidal activity, e.g., from 8.4% ± 6.9% to 91.5% ± 5.8% with BAL fluid sample 1 (BAL 1) ($P = 0.0004$) (Fig. 2). The NE concentration range of the BAL fluid samples was 46.4 μg/ml to 193.3 μg/ml. The incubation of pro-WMR with two non-CF BAL fluid samples from patients with sarcoidosis (in which no NE activity was detected) resulted in no cleavage of the peptide after 3 h of incubation. As expensive D-amino acids represent a potential economic barrier to the large-scale production of peptide therapeutics, an all-L-amino-acid version of pro-WMR was synthesized. However, although both the D- and L-forms of pro-WMR had comparable high MICs (Table 1), when they were incubated with purified NE or CF BAL fluid, the region of the L-amino acid active sequence as well as the promoiety was cleaved. In addition, the bactericidal activity against PAO1 was not increased with the addition of NE (see Fig. S2 in the supplemental material); in contrast, that of pro-D-WMR was increased with the addition of NE.

**Pro-WMR demonstrates low cytotoxicity against human cells.** Both pro-WMR and the cleavage product, AAG-WMR, displayed low cytotoxicities against CF bronchial epithelial (CFBE) cells at an $IC_{50}$ of over 300 μM, but solubility issues precluded the extension of the concentration range beyond 300 μM for pro-WMR (Table 2). The released pro-moiety (synthesized from an Ala-Wang resin), EEEEA-OH with an acetyl (Ac) group at the N terminus (Ac-EEEEA-OH), also demonstrated no cytotoxicity. Similarly, the $IC_{50}$s for CF tracheal epithelial (CFTE) cells were >600 μM for AAG-WMR and >300 μM for pro-WMR. These were higher than the $IC_{50}$ for pro-P18, the previous least toxic pro-AMP that was active in BAL fluid, with $IC_{50}$s being 55.9 μM and 4.7 μM for pro-P18 and AAG-P18, respectively.

Since the pro-AMPs are designed for cleavage by a neutrophil-derived enzyme, the toxic effects of the cleaved active peptides against purified neutrophils was investigated. At 3 h of incubation, the $IC_{50}$ for AAG-P18 was 9.2 μM and that for AAG-WMR was >300 μM (Table 2).

**Pro-AMPs do not stimulate IL-8/IL-6 release.** At sub-$IC_{50}$s of pro-WMR and AAG-WMR (up to 100 μM), negligible levels of the proinflammatory cytokines interleukin-8 (IL-8) and IL-6 were released from CFBE cells compared to the levels of the positive control (LPS) released. Similarly, no release was observed with the propeptides and cleaved peptides of HB43 and P18 (Fig. 3).

**Prodrug modification reduces the *in vivo* toxicity of active peptides.** To compare the toxicities of propeptides and active peptides, C57BL/6 mice were treated twice intratracheally with 50 μg of peptide and sacrificed the next morning. Of the four mice treated with AAG-P18, three died (Table 3). On the other hand, all mice treated with pro-P18 survived. However, these mice displayed significant weight loss compared to that for the PBS-treated control mice (5.0% ± 0.7% and 0.4% ± 1.3%, respectively; $P = 0.018$) and raised lung neutrophil numbers ($3.8 \times 10^5 \pm 9.5 \times 10^4$ and $2.2 \times 10^3 \pm 6.4 \times 10^2$, respectively; $P = 0.0073$). Both groups of mice treated with AAG-WMR and pro-WMR survived. However, mice treated with AAG-WMR displayed significant weight loss compared to that for mice treated with pro-WMR (8.0% ± 1.6% and 0.4% ± 0.5%, respectively; $P = 0.0037$) (Fig. 4). Lung neutrophil numbers were also significantly increased in mice treated with pro-WMR compared to those in the PBS-treated control mice, and the trend

**TABLE 2** $IC_{50}$s of parent AMPs, cleaved AMPs, and pro-AMPs for CFBE and CFTE cell lines and healthy neutrophils

| Peptide | Sequence[a] | $IC_{50}$ (μM) | | |
|---|---|---|---|---|
| | | CFBE cells | CFTE cells | Neutrophils |
| AAG-WMR | AAGwglrrllkygkrs-$NH_2$ | >300 | >600 | >300 |
| Pro-WMR | Ac-EEEEAAAGwglrrllkygkrs-$NH_2$ | >300 | >300 | ND[b] |
| L-Pro-WMR | Ac-EEEEAAAGWGLRRLLKYGKRS-$NH_2$ | >300 | ND | ND |
| AAG-P18 | AAGkwklfkklpkfhlhlakkf-$NH_2$ | 35.5[c] | 4.7 | 9.2 |
| Pro-P18 | Ac-EEEEAAAGkwklfkklpkfhlhlakkf-$NH_2$ | 77.3[c] | 55.9 | ND |
| Pro-moiety | Ac-EEEEA-OH | >300 | ND | ND |

[a] Amino acids in uppercase letters are L-amino acids, and those in lowercase letters are D-amino acids.
[b] ND, not determined.
[c] Data are from reference 16.

was for increases in lung neutrophil numbers in mice treated with AAG-WMR ($P = 0.066$).

**Prodrug modification reduces the *in vivo* immunogenicity of AAG-WMR.** Cytokine analysis was carried out on the BAL fluids of the mice. The release profile was variable, but a statistically significant increase in cytokine levels that correlated with the weight loss results was observed. TNF-α release was increased in mice treated with AAG-WMR compared to that in PBS-treated control mice (23.2 ± 5.4 pg/ml versus 3.0 ± 1.8 pg/ml; $P = 0.01$). This increase was not seen with pro-WMR treatment. There was a trend for increased cytokine release with pro-P18 treatment, but this did not reach statistical significance (for TNF-α, $P = 0.571$).

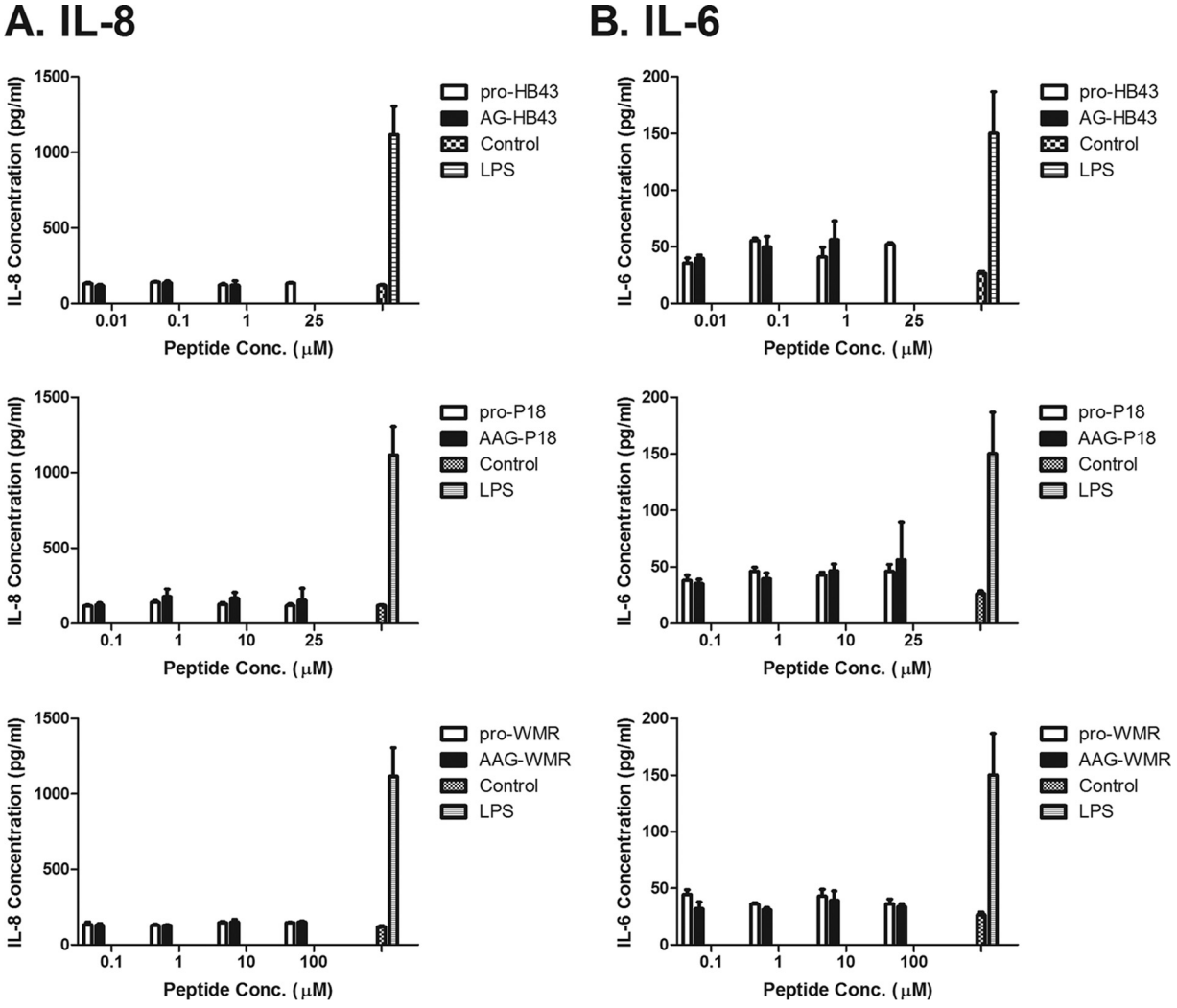


**FIG 3** Release of the IL-8 (A) and IL-6 (B) cytokines from CFBE cells in response to incubation with propeptides and active peptides at sub-$IC_{50}$s for 24 h. The LPS concentration was 50 μg/ml, and the control represents cells alone. The values shown are the means ± SEMs from three independent experiments.

**TABLE 3** Mouse survival after two intratracheal treatments with 50 μg of peptide

| Peptide | No. of mice that survived (no. of mice that died) |
|---|---|
| PBS control | 4 (0) |
| AAG-WMR | 4 (0) |
| Pro-WMR | 4 (0) |
| AAG-P18 | 1 (3) |
| Pro-P18 | 4 (0) |

The amounts of TNF-α and KC released in response to AAG-WMR were significantly increased compared to the amounts released in response to pro-WMR (28.2 ± 6.5 pg/ml versus 8.3 ± 3.6 pg/ml; $P = 0.0365$ for the latter) (Fig. 5).

## DISCUSSION

We previously demonstrated, using the pro-AMPs pro-HB43 and pro-P18, how an oligoglutamic acid modification could be used to target the activity of AMPs while limiting the cytotoxicity (16). However, the pro-AMPs of that study were still not optimal in terms of cytotoxicity. This prompted a search for novel sequences from the large AMP library based on desirable characteristics identified previously and additional properties, such as negligible immunogenicity. The two candidates selected, WMR and WR12, synthesized as pro-AMPs, produced similar cleavage patterns in response to NE, as seen previously (16), and displayed greater salt tolerance than the previous group of cleaved AMPs. This is a favorable characteristic for an AMP intended to be an anti-infective for CF patients, as CFTR dysfunction has been linked to increasing salt concentrations in the ASL (15), although this remains the subject of debate (36–39). In addition, high salt tolerance would facilitate the delivery of these AMPs with hypertonic saline, the inhalation of which reduces airway mucus plugging in mice with CF-like lung disease and improves lung function in patients with CF (40–42). However, while the cleaved peptides were more active against *P. aeruginosa* than the pro-AMPs, it must be noted that their activity tended to be less than that previously reported in the literature; i.e., MIC values were 64 μg/ml, whereas they were 3.3 μg/ml for L-WMR (26). The differences in MICs may be due to the use of different strains and assay conditions. Future work may focus on achieving further improvements in activity.

The synthesis of pro-WR12 was challenging, requiring multiple coupling cycles for each amino acid after the active sequence and resulting in low yields. This was in contrast to the synthesis of pro-WMR, which was less complex. Given these restraints on pro-WR12 synthesis, further studies were carried out on pro-WMR only. In 25% (vol/vol) CF BAL fluid, pro-WMR performed better than both pro-HB43 and pro-P18, with nearly full bactericidal activity being achieved in BAL fluid but little activity being achieved in its absence, characteristics not seen with the other AMPs tested (16). This is significant because components of BAL fluid, such as proteases, mucins, and extracellular DNA, may inactivate other AMPs (40, 43, 44). The complete cleavage of pro-WMR to the active AMP was observed after 3 h of incubation with 50% (vol/vol) CF BAL fluid, and no conversion was observed in non-CF BAL fluid (which contained no NE activity). This demonstrates the specific activation afforded by the pro-moiety and linker.

With regard to the active sequence itself, as the cost of production of peptide drugs is greatly increased by the use of nonproteinogenic amino acids, such as the D-amino acids used here, their necessity in the design was investigated. While all-L-pro-WMR provided reduced cytotoxicity and antimicrobial activity (Tables 1 and 2), it was cleaved in its active sequence and deactivated by NE both in the purified form and in CF BAL fluid and therefore is unsuitable for use in this disease model. The HPLC and MALDI-TOF MS analyses indicated multiple cleavage sites, ruling out the simple substitution of one or two amino acids with D-amino acid isomers. This would have represented an alternative approach to reducing costs and the issue of increased side effects with a prolonged half-life (45). Degradation in CF patient samples has previously been seen with the AMP P-113, and similarly, the D-peptide was much more stable (46). This is, to our knowledge, the first investigation into the necessity of D-amino acids in a pro-AMP for CF. It appears that an all-D-amino acid active sequence is essential to survive the challenging proteolytic conditions.

Cytotoxicity is one of the major issues that have limited the

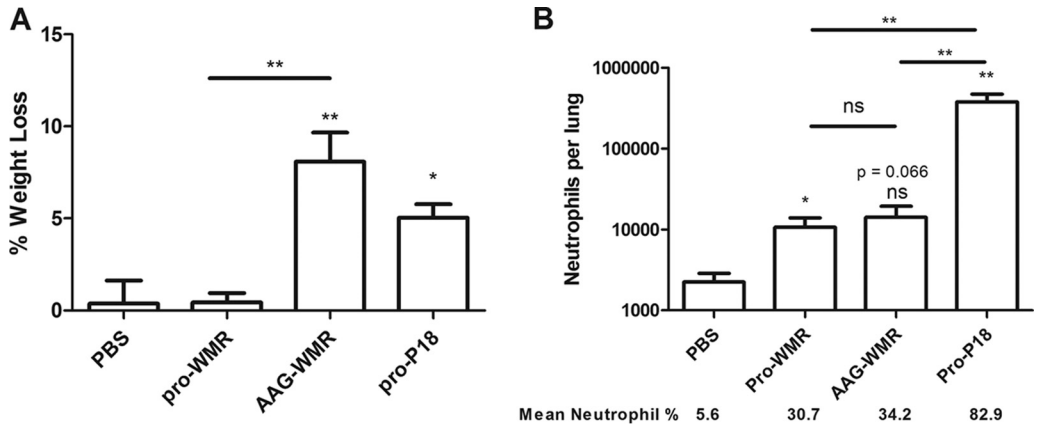


**FIG 4** The percent weight loss measured after 24 h (A) and the lung neutrophil counts measured after treatment (B) of C57BL/6 mice in response to two doses (morning and evening) of 50 μl PBS as a control or 50 μg AMPs ($n = 4$ mice for each group). Statistical analyses were carried out using an unpaired two-tailed *t* test. *, $P < 0.05$ compared to the PBS-treated control mice; **, $P < 0.01$ compared to the PBS-treated control mice. Lines show where comparisons between treatment groups have been made. ns, not significant (with *P* values being given above the bars in some cases). AAG-P18 was not included, as three of four mice died before lung lavage.

progress of AMPs as therapeutics. Despite the improvements previously seen with HB43 and P18 after pro-AMP modification, some cytotoxic effects were evident on the basis of their low $IC_{50}$s against CFBE cells (50.8 μM and 77.3 μM, respectively) (16). Their toxic effect on neutrophils, from which large amounts of the target enzyme are derived, was also unknown and to our knowledge has not been investigated with AMPs before. Pro-WMR shows superiority to the previous group of pro-AMPs, exhibiting a lower level of cytotoxicity against CFBE cells, CFTE cells, and neutrophils. This is in agreement with the low level of cytotoxicity against Vero cells originally seen with L-WMR by others (25, 26).

The cleaved peptide AAG-WMR was not as active against *P. aeruginosa* as AG-HB43 or AAG-P18 (16), but the reduced toxicity compensated for this. For example, it was observed in this study that AAG-P18 demonstrated a high level of toxicity against neutrophils with an $IC_{50}$ of 9.2 μM (23 μg/ml). It is therefore likely that, upon delivery to the CF patient lung, neutrophils would be subjected to a high concentration of the active peptide and a large proportion would be killed. While CF is a neutrophil-dominated disease and the resultant high levels of NE contribute to morbidity (10, 11), it may be unfavorable to kill immune cells when a patient is suffering from a potentially severe infection. If the mechanism of cell death is necrosis, this could lead to the release of extracellular DNA in patients with CF, which increases mucus viscosity and facilitates bacterial attachment (13).

The immunomodulatory properties of many endogenous AMPs, such as LL-37, are well documented (47); however, the effects of exogenous peptides on immune function are less clear. Although the rationally designed innate defense regulator (IDR) AMPs are devoid of antimicrobial activity, they demonstrate anti-inflammatory effects and are protective against infection. The mechanism may involve interaction with intracellular targets or via a direct receptor interaction (48). One might expect the D-AMPs, such as those used here, not to interact with receptors and have immunomodulatory effects. Nonetheless, it has been demonstrated that D-LL-37 can stimulate the release of far larger amounts of IL-8 from keratinocytes than L-LL-37, arguing against the necessity of structure-specific binding to receptors for cytokine release (49). At concentrations below their $IC_{50}$s (up to 100 μM for the WMR peptides), neither pro-AMPs nor cleaved AMPs induced significant IL-6 or IL-8 release from CF bronchial epithelial cells. This is a desirable characteristic, as IL-8 is a potent chemoattractant for neutrophils (50). The lack of a proinflammatory cytokine response to the pro-AMPs is probably due to the lack of a response to the active AMPs. However, if an immunomodulatory AMP was modified, there is the possibility that the addition of the pro-moiety could reduce the effects, and this should be taken into account when investigating the effects of pro-AMP modification. For example, it has been observed with one AMP that PEGylation can reduce its ability to inhibit LPS-induced NF-κB activation of macrophages (51).

While prodrug modification reduced the antimicrobial activity of AAG-WMR, its lack of observed cytotoxicity made the benefits of the modification difficult to determine *in vitro*. However, the benefits of pro-AMP modification were evident *in vivo*. The 100% survival of pro-P18-treated mice compared to the 25% survival of AAG-P18-treated mice demonstrates the benefits of the pro-AMP model and is consistent with the reduced cytotoxicity noted previously (16). Consistent again with the *in vitro* cytotoxicity outlined in Table 2, there was less lung disease in both pro-WMR- and AAG-WMR-treated mice than pro-P18-treated mice, on the basis of lower lung neutrophil counts. The absence of weight loss with pro-WMR treatment also illustrates the benefits of the prodrug model. CF patients undergoing an acute pulmonary exacerbation frequently experience acute weight loss. In mice, this phenomenon has been demonstrated to be potentially related to increased pulmonary inflammation, and the BAL fluid levels of the cytokines KC, TNF-α, and macrophage inflammatory protein 2 have all been shown to correlate with weight loss in mice (52). The observed increase in cytokine levels in response to the peptides supports this, especially when one considers that there was no observed significant increase in cytokine levels with pro-WMR treatment. An increase in cytokine release in CFBE cells was not observed *in vitro* with AAG-WMR treatment but was observed with treatment with its prodrug *in vivo* (Fig. 5). The *in vivo* increase in KC and TNF-α levels may not be the result of direct immunostimulation but could, alternatively, be the indirect result of epithelial damage that the pro-AMP modification protects the lungs from. These cytokines have the potential to exacerbate inflammation in patients with CF. TNF-α increases neutrophil che-

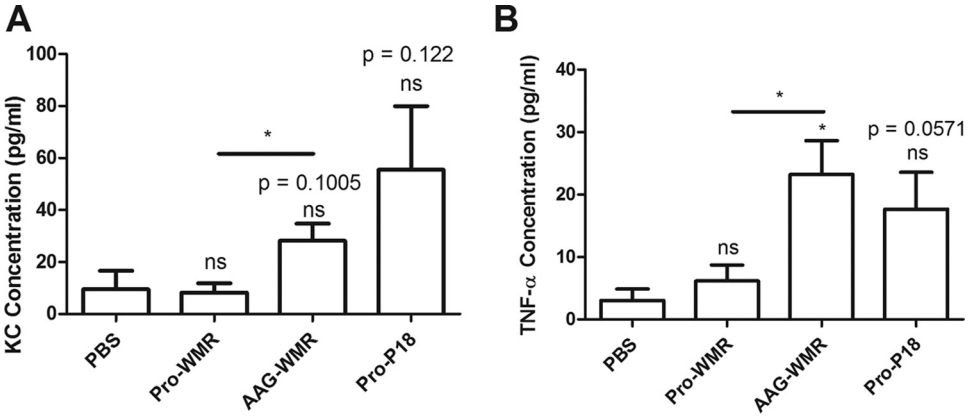


**FIG 5** Mouse BAL fluid levels of KC (A) and TNF-α (B) measured after 24 h in response to two doses (morning and evening) of 50 μl PBS as a control or 50 μg AMPs ($n = 4$ mice for each group). Statistical analyses were carried out using an unpaired two-tailed *t* test. *, $P < 0.05$ compared to the PBS-treated control mice. Lines show where comparisons between treatment groups have been made. ns, not significant (with *P* values being given above the bars in some cases). AAG-P18 was not included, as three of four mice died before lung lavage.

motaxis, adhesion, and production (53), while KC is also a neutrophil chemoattractant (35). There is no structural analogue of IL-8, a potent human neutrophil chemoattractant, in mice; therefore, it could not be analyzed here (54). The *in vivo* results illustrate the benefit of delivering AMPs as a prodrug, with the prevention of mortality with the P18 series and the prevention of weight loss and cytokine release with the less toxic WMR series. In total, the *in vivo* results are consistent with *in vitro* data, with pro-WMR being the least toxic, with the toxicity increasing for AAG-WMR and then pro-P18, and, finally, with AAG-P18 being the most toxic. Toxicity has been noted before when AMPs are delivered to the lung to treat infection. The delivery of LL-37 and IDR-1 at the same time as methicillin-resistant *Staphylococcus aureus* (MRSA) has been used to ameliorate the lung disease induced by the bacteria. However, at higher doses (50 to 66 μg/mouse), the protective effects of both peptides were lost. The survival time of the mice was also reduced compared to that of the MRSA-infected control mice not treated with peptide, indicating a degree of host toxicity (55). We demonstrate here how the prodrug model can be used to circumvent these issues of toxicity and that many of the shortcomings of AMP use in patients with CF, such as protease lability and cytotoxicity, are not insurmountable.

The increasing threat of antimicrobial resistance and the unique challenges in treating *P. aeruginosa* respiratory infections in patients with CF emphasize the need for new therapeutic approaches. While further research is required to advance the use of pro-AMPs as an alternative approach in this setting, with perhaps further improvements in activity, we believe that pro-AMPs have significant potential.

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