

## De novo-derived cationic antimicrobial peptide activity in a murine model of *Pseudomonas aeruginosa* bacteraemia

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**Objectives:** We describe the antimicrobial activity against *Pseudomonas aeruginosa* of the *de novo*-derived antimicrobial peptide WLBU2 in an animal model of infection.

**Methods:** For this study, an intravenous (iv) model of *P. aeruginosa* infection was established. The minimum lethal murine dose of *P. aeruginosa* strain PA01 was determined to be  $3 \times 10^7$  cfu when bacteria were administered iv. Increasing concentrations of WLBU2 were instilled either prior to or following PA01 septic exposure.

**Results:** For the mice given peptide post-bacterial infection, in the 1 mg/kg group, nine of nine animals died because of *Pseudomonas* sepsis; in the 3 mg/kg group, only one of nine succumbed to infection and in the 4 mg/kg group, all mice were protected ( $P < 0.0001$ ). Similar results were obtained when WLBU2 was given 1 h prior to *Pseudomonas* infection.

**Conclusions:** Although the therapeutic window in this model is narrow, the results nonetheless provide encouraging evidence for WLBU2 as a potential prophylactic or treatment of bacterial infection.

Keywords: antimicrobial peptide, WLBU2, sepsis

### Introduction

Despite the development of safe and potent antibiotics, serious bacterial infections remain a worldwide health priority. An increasing concern is the emergence of multidrug-resistant (MDR) pathogens and their role as opportunistic pathogens.<sup>1</sup> Resistant *Pseudomonas aeruginosa* has become particularly important in the setting of immunocompromised, cystic fibrosis, septic, burn and ventilated patients. Owing to the significant antibiotic resistance in MDR pathogens,<sup>2</sup> there is a critical need for the development of more effective antimicrobials with unique bactericidal mechanisms to overcome resistance.

We have developed a novel class of engineered cationic antimicrobial peptides (eCAPs),<sup>3</sup> which originate from discrete segments of the lentiviral transmembrane protein cytoplasmic tail. The prototype peptide is LLP1 that is derived from the C-terminal 28 residues encoded by HIV-1 gp41. A series of peptides based on a six amino acid residue motif that retains the cationic amphipathic character of the peptide were designed.<sup>4</sup> These hexamers were designed by positioning key amino acids,

Arg, Val or Trp, to maintain a cationic and hydrophobic face when modelled as an  $\alpha$ -helix. It was reasoned that these hexapeptides could be oligomerized through solution-phase chemistry, significantly decreasing the cost of synthesis. Using these principles, a series of *de novo* antimicrobial peptides were designed<sup>4</sup> and a lead 24-residue peptide, WLBU2, was identified. To characterize the toxicity of WLBU2 under these conditions, red blood cells, white blood cells and human skin fibroblasts co-cultured with *P. aeruginosa* were exposed to WLBU2 and viability assessed.<sup>4</sup> To further evaluate toxicity, an intraperitoneal (ip) mouse model with *P. aeruginosa* infusion was established and WLBU2 given intravenously (iv) to prevent the progression of infection.<sup>4</sup>

To investigate the potential role of WLBU2 as a prophylactic or therapeutic agent, an iv *P. aeruginosa* sepsis mouse model was developed to control the systemic level of bacterial infusion into the circulatory system. Systemic administration of WLBU2, both at prophylactic and at therapeutic time points, increased the survival of *P. aeruginosa*-infected mice by preventing progression to bacterial sepsis.

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## Materials and methods

### Peptide synthesis

The engineered peptide WLBU2 (RRWVRRVRRWVRRVVRVVRWVRR) was synthesized, purified and quantified, as previously described.<sup>3–5</sup>

### Bacteria and killing assays

*P. aeruginosa* PAO1 was used throughout these studies. Bacterial suspensions ( $1 \times 10^6$  cfu/mL) in 10 mM potassium phosphate buffer containing 150 mM NaCl (PBS), pH 7.2 were incubated with 2-fold dilutions of WLBU2 at 37°C for 1 h. Serial peptide dilutions were performed and plated on tryptic soy agar (TSA; Difco, Detroit, MI, USA). Surviving colonies were counted the next day to determine the MBC, defined as the molar concentration of peptide reducing the viable bacteria within a suspension by three orders of magnitude.

### Distribution of bacteria in fluids and organs

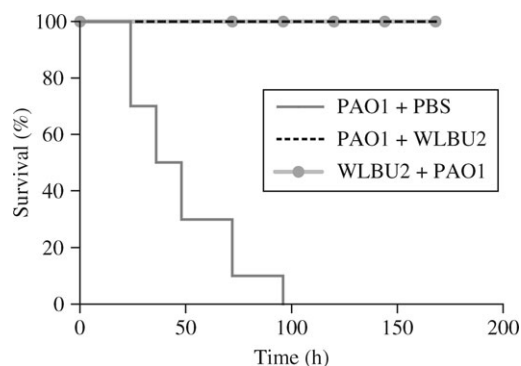
Quantitative blood cultures were performed to determine bacterial loads over the course of the infection. Blood samples were obtained from the tail vein by aseptic percutaneous puncture 1–24 h after bacterial challenge and serially diluted. A 0.1 mL volume of each dilution was plated on TSA and incubated at 37°C overnight for enumeration of developed colonies. Toxicity was evaluated on the basis of the presence of peptide-related adverse effects such as signs of inflammation, weight loss and presence of bacteria in the blood and tissues. Throughout the course of the infection or at the disease endpoint, animals were euthanized and tissues weighed and homogenized using 70  $\mu$ m cell strainers to determine bacterial cfu/g tissue.

### iv bacterial inoculation and iv antibacterial therapy

Swiss Webster mice (25–30 g) (Taconic, Germantown, NY, USA) were maintained and procedures performed according to the protocol approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh. Suspensions of mid-log phase *P. aeruginosa* were centrifuged at 2000 g for 10 min. Supernatants were discarded and the bacteria suspended and diluted in sterile PBS to achieve a concentration of  $\sim 2\text{--}4 \times 10^8$  cfu/mL. Mice were injected iv with 0.1 mL of the bacterial suspensions, estimated as the minimum lethal dose. The animals were then randomized to receive iv PBS (control group), or 1, 1.5, 3 and 4 mg/kg WLBU2  $\sim 60$  min after bacterial challenge. The animals in each group, which included 7–11 mice, were returned to individual cages and subsequently monitored for up to 7–10 days for survival. The endpoints of the study were indicated either by 7–10 days of survival or by complete absence of motility as a sign of terminal illness. For the prophylactic portion, animals were randomized to receive iv PBS versus a 3 mg/kg WLBU2 bolus 1 h prior to infusion of bacterial suspension as described earlier. The group consisted of six mice and was monitored as described earlier.

### Statistical analysis

Murine survival data for either the WLBU2-uninfected or WLBU2-infected group were analysed using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego, CA, USA). Kaplan–Meier survival analysis was performed and log-rank test was used to compare survival between groups. Significance was accepted at a *P* value of less than 0.05.



**Figure 1.** Therapeutic and prophylactic protection by WLBU2 in the bacterial sepsis model. Kaplan–Meier survival analysis comparing 1 h pre-infection infusion of WLBU2 (WLBU2+PAO1), 1 h post-infection infusion of WLBU2 (PAO1+WLBU2) and a PBS control group.

## Results

### Murine iv pseudomonal sepsis model

The minimum iv-administered *P. aeruginosa* lethal dose (PLD) was determined by establishing the lowest iv bacterial dose ( $3 \times 10^7$  cfu) leading to 100% mortality (data not shown). After administration of an iv PLD ( $3\text{--}4 \times 10^7$  cfu), 11 mice were treated 1 h post-infection with 3 mg/kg WLBU2 (PAO1 + WLBU2) or PBS. At the experimental endpoint, 100% survival was observed for WLBU2-treated mice. Infusion 1 h pre-infection of 11 mice with 3 mg/kg WLBU2 (WLBU2 + PAO1) or PBS (PBS + PAO1) led to 100% survival for WLBU2-treated mice. A log-rank test reveals a *P* value of less than 0.0001 when comparing survival of WLBU2-treated with that of PBS-treated mice. In comparison with the PBS-treated mice, no lethality was observed in prophylactically and therapeutically treated groups (Figure 1). These results provide evidence for a potential prophylactic and therapeutic role of WLBU2 in the treatment of *P. aeruginosa* sepsis.

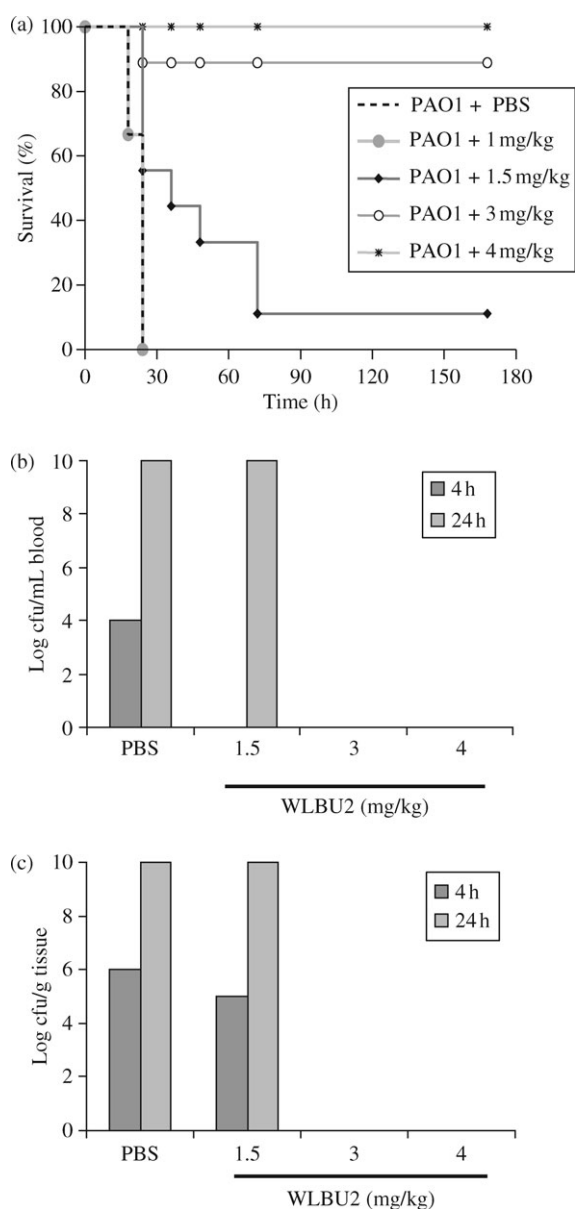
### Murine dose dependence of WLBU2 protection from bacterial sepsis

Infected mice (nine per group) were treated therapeutically after 1 h with 0, 1, 1.5, 3 and 4 mg/kg peptide and monitored for 7 days post-treatment. WLBU2 effectively eradicated the infection at a minimum of 3 mg/kg (Figure 2a), with one fatality out of nine mice treated. These data demonstrate dose dependence of WLBU2 protection from *P. aeruginosa* sepsis. Mice were also euthanized at 4 and 24 h post-treatment to determine bacterial loads in the blood (Figure 2b) and kidney (Figure 2c). In the blood, the bacterial load decreased from  $10^4$  cfu/mL to 0 cfu/ml after treatment with 3 or 4 mg/kg WLBU2. Similar results were obtained for per gram of tissue when the kidney was sampled. These experiments show a dose dependence of WLBU2 treatment. This suggests that WLBU2 can play a therapeutic role in helping to control the bacterial infection in peptide-treated mice.

## Discussion

The role of antimicrobial peptides in innate immunity has been long established through various studies of the microbial killing

## In vivo engineered cationic antimicrobial activity



**Figure 2.** Survival and bacterial loads vary with peptide doses. (a) Kaplan–Meier survival analysis comparing varying doses of WLBu2 versus a PBS control group. (b) Log cfu/mL blood and (c) log cfu/g tissue at 4 and 24 h post-PAO1 infusion.

mechanisms of neutrophils and macrophages.<sup>6</sup> However, it was not until the last two decades that CAPs have been seriously considered as a potential therapeutic source. This is even more critical because of emerging multiple drug resistance among bacteria. Although numerous CAPs with potent antibacterial efficacy *in vitro* have been described, only few display significant activity in animal models.<sup>7,8</sup> An important explanation is that CAP activity is suppressed in biological environments. To address this, a series of studies were initiated to develop *de novo* CAPs for clinical applications.<sup>4,9,10</sup> From these studies, we have demonstrated that the peptide WLBu2 retained its activity against *P. aeruginosa* in the presence of physiological concentrations of monovalent and divalent cations, human serum and

whole blood.<sup>4</sup> These studies warranted further investigations of WLBu2 efficacy *in vivo*.

As can be seen from the experiments (Figure 1), the prophylactic timing of the dose for WLBu2 served a protective role against the infusion of PLD. This dose of 3 mg/kg was as effective as the two high treatment doses for the therapeutic experiments; therefore, the prophylactic window is within the 1 h prior to exposure of an infectious agent. As a therapeutic agent, WLBu2 at concentrations of 3 and 4 mg/kg given 1 h post-infusion of PLD demonstrated increased survival rates when compared with the lower concentration group as well as the PBS control group. This suggests that, at lower concentration, WLBu2 is not able to control the development of sepsis. Our maximal tolerated dose iv was 14 mg/kg and our effective therapeutic dose for 100% survival is 4 mg/kg. Therefore, our therapeutic index is 3.5. This index would be slightly more favourable than similar antimicrobial peptides (e.g. colistin) currently used in clinical scenarios with MDR pathogens.

Taken together, this study demonstrates the successful development of a *P. aeruginosa* sepsis model in the context of a competent immune system. Using this model, we show that the *de novo*-derived CAP was prophylactic and therapeutic against *P. aeruginosa* bacteraemia in mice. These results provide fundamental information that may be useful for evaluating *in vivo* efficacy of other CAPs. Further, the data underscore the need for comparative studies of WLBu2 to establish the potential of these proposed eCAPs for therapeutic and/or prophylactic antimicrobial advancement.

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## Transparency declarations

None to declare.

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