Journal of Medical Microbiology (2016), **65**, 188–194 DOI 10.1099/jmm.0.000209

Novel engineered cationic antimicrobial peptides display broad-spectrum activity against *Francisella tularensis*, *Yersinia pestis* and *Burkholderia pseudomallei*

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Received 24 August 2015
Accepted 14 December 2015

Broad-spectrum antimicrobials are needed to effectively treat patients infected in the event of a pandemic or intentional release of a pathogen prior to confirmation of the pathogen's identity. Engineered cationic antimicrobial peptides (eCAPs) display activity against a number of bacterial pathogens including multi-drug-resistant strains. Two lead eCAPs, WLBU2 and WR12, were compared with human cathelicidin (LL-37) against three highly pathogenic bacteria: *Francisella tularensis*, *Yersinia pestis* and *Burkholderia pseudomallei*. Both WLBU2 and WR12 demonstrated bactericidal activity greater than that of LL-37, particularly against *F. tularensis* and *Y. pestis*. Only WLBU2 had bactericidal activity against *B. pseudomallei*. WLBU2, WR12 and LL-37 were all able to inhibit the growth of the three bacteria *in vitro*. Because these bacteria can be facultative intracellular pathogens, preferentially infecting macrophages and dendritic cells, we evaluated the activity of WLBU2 against *F. tularensis* in an *ex vivo* infection model with J774 cells, a mouse macrophage cell line. In that model WLBU2 was able to achieve greater than 50 % killing of *F. tularensis* at a concentration of 12.5 μM. These data show the therapeutic potential of eCAPs, particularly WLBU2, as a broad-spectrum antimicrobial for treating highly pathogenic bacterial infections.

INTRODUCTION

Biodefence agents are pathogenic organisms or toxins that pose a severe threat to public health and can be used against humans, animals or plants for terrorist purposes. These pathogens include the Gram-negative bacteria *Francisella tularensis*, *Burkholderia pseudomallei* and *Yersinia pestis*. Due to their ability to cause fatal disease when inhaled, the use or even threatened use of such organisms can produce widespread social disruption. Many of the viruses and bacteria considered to be potential biological weapons have very similar initial prodromes with 'influenza-like' symptoms. A delay in diagnosis can be a critical determinant in whether the patient will survive the infection. While diagnostics are an area of intense focus, there would currently be a considerable delay before initiation of treatment. A priority area of research is the development of broad-spectrum countermeasures that could be utilized prior to confirmation of the diagnosis to rapidly reduce pathogen load and improve the odds of saving the patient.

Abbreviations: CAP, cationic antimicrobial peptide; MDR, multi-drug resistant; LPS, lipopolysaccharide.

Cationic antimicrobial peptides (CAPs) are amphipathic peptides of 12–50 aa with a net positive charge. They are ubiquitous peptides naturally found in all living species and are known to be active components of the innate immunity against infectious pathogens, including viruses and bacteria (Hancock & Chapple, 1999). In response to infectious pathogens, CAPs can be released from macrophages, neutrophil granules, or in secretions from epithelial cells found in the skin or mucosal tissues. CAPs can act against a wide range of targets including Gram-positive and Gram-negative bacteria, fungi and parasites; the essential component in these diverse targets is a negatively charged plasma membrane (Boman, 1995). Therefore, normal eukaryotic cells have a relatively high resistance due to the preference of CAPs for negatively charged membranes. The mechanism by which CAPs function is not fully understood, although studies have implicated the electrostatic interaction between the peptides and the lipid molecules on the bacterial membrane. When compared with other antibiotics, CAPs are able to kill bacteria rapidly, within 30 to 180 s, limiting the bacterium's ability to develop resistance against these peptides (Ntwasa, 2012). Therefore, CAPs are considered a good candidate for use against multi-drug resistant (MDR) bacteria.

Although natural CAPs have a wide spectrum of action against pathogens, their activity strongly depends on the environment in which they are acting. The optimal potency of each peptide is only achieved in specific environmental conditions and readily lost when subjected to a different setting (Goldman *et al.*, 1997). Therefore, to be able to utilize their antimicrobial activity to the fullest, we have designed *de novo*-synthesized engineered CAPs.

Studies of the lentivirus lytic peptide 1 (LLP1) domain of HIV-1 gp41 demonstrated that it had remarkable antimicrobial properties (Miller *et al.*, 1993). That work demonstrated that an α-helical amphipathic CAP, using arginine residues on the polar side and valine residues on the non-polar side, greatly improved the potency and selectivity of LLP1. Further studies of the influence of length and helical structure on activity led to development of a series of rationally engineered peptides designated WLBU (Tencza *et al.*, 1999). WLBU peptides are composed of arginine in the hydrophilic face and valine and tryptophan residues in the hydrophobic face arranged to form idealized amphipathic helices. Moreover, WLBU studies demonstrated that tryptophan substitution significantly increased antimicrobial activity in environments such as serum and blood. Detailed studies demonstrated that WLBU2, a 24-mer, had the minimum length required for optimal activity against a wide range of bacteria including methicillin-resistant *Staphylococcus aureus* (MRSA), and MDR strains of *Klebsiella pneumoniae* and *Acinetobacter baumannii*.

To further minimize length in optimally active eCAPs, a second series of peptides (the WR series) were developed with only arginine in the hydrophilic face and tryptophan in the hydrophobic face (Deslouches *et al.*, 2005b). *In vitro* studies demonstrated that WR12, consisting of only 12 amino acids, had better activity against bacteria than longer peptides in the WR series and at least comparable to WLBU2 (Deslouches *et al.*, 2005a). These data suggested that WLBU2 and WR12 could serve as a broad-spectrum countermeasure against a wide range of bacteria including those that represent potential biological weapons.

In this study we compared the potency of WLBU2 and WR12 with that of the natural peptide LL37 using *in vitro* and *ex vivo* evaluations against *F. tularensis*, *Y. pestis* and *B. pseudomallei*. The results demonstrated that *de novo* synthesized peptides outperformed LL37 against all three pathogens.

METHODS

Biosafety. All experiments using *F. tularensis*, *B. pseudomallei* or *Y. pestis* were performed in the biosafety level 3+ Regional Biocontainment Laboratory (RBL) at the University of Pittsburgh. Powered air purifying respirators were worn for respiratory protection, and all work was conducted in a class II biosafety cabinet using Vesphene IIse (diluted 1:128; Steris) as a disinfectant. All three bacteria are tier 1 select agents and required Department of Justice approval, an approved security risk assessment with the Centers for Disease Control's Division of Select Agents & Toxins, and a Tier 1 suitability assessment for access to the pathogens.

Bacteria. All of the bacteria used in these studies were obtained from the Biodefence and Emerging Infections Research Resources Repository, Manassas, VA, USA. All bacteria were passaged a single time in culture and frozen at −80 °C prior to use in these studies. For all three bacteria, a similar process was followed for preparation of the bacteria to use in these experiments. Bacteria were grown first on solid media prior to an overnight culture in 25 ml liquid broth media using a baffled, vented 125 ml polycarbonate Erlenmeyer flask incubated in a bacterial shaker set at 200 r.p.m. *Francisella tularensis* SchuS4 strain was grown on cysteine heart agar (CHA Becton Dickinson, La Jolla, CA) for 2 days at 37 °C prior to culture in brain heart infusion broth (BHI; Becton Dickinson, La Jolla, CA) for 17–19 h at 37 °C (Faith *et al.*, 2012). *Burkholderia pseudomallei* strain 1026b was grown on Luria–Bertani (LB; Becton Dickinson, La Jolla, CA) Agar for 1 day at 37 °C and then cultured overnight in BHI for 11 h at 37 °C. *Yersinia pestis* strain CO92 was grown on tryptic soy agar (TSA; Becton Dickinson, La Jolla, CA) for 2 days at 30 °C and then cultured in heart infusion broth (Becton Dickinson, La Jolla, CA) for 24–27 h at 37 °C.

Peptides. Peptides were synthesized using standard Fmoc (9-fluorenylmethoxycarbonyl chloride) synthesis protocols (Genscript). Synthetic peptides were characterized and purified by reverse-phase HPLC on Vydac C18 or C4 columns (The Separations Group). The identification of each was established by mass spectrometry (Electrospray Quatro II triple quadruple mass spectrometer; Micromass). Peptide concentrations were determined using a quantitative ninhydrin assay. Details of the peptides and their sequences are in Deslouches *et al.*, 2005b.

Bacterial killing assay. Bacteria cultured overnight were diluted to a concentration of 1×10^6 c.f.u. ml^{-1} in phosphate buffer saline or potassium phosphate buffer and 100 µl was placed per well in the first column of a 96-well plate. Twofold dilutions of peptides (concentration of 1.5 µM to 100 µM) were added to the bacteria, and the plate was incubated for 60 min at 37 °C. Serial dilutions were plated on agar plates to determine surviving bacteria. Colonies were counted following an appropriate incubation time (24 h for *B. pseudomallei*; 48–72 h for *F. tularensis* and *Y. pestis*) to determine the reduction in bacterial count.

Growth inhibition assay. Bacterial suspensions (concentration of 1×10^6 c.f.u. ml^{-1}) in growth media were incubated with twofold dilutions of peptides (concentration of 1.5 µM to 100 µM) in a 96-well plate for 24 to 48 h at 37 °C. After incubation, the optical density of each well was measured at 600 nm using a spectrophotometer (FluoStar Omega, BMG LabTech). Per cent inhibition was calculated from the difference in optical density measured.

***Ex vivo* infection assay.** For *ex vivo* infection with *F. tularensis*, a standardized assay using a mouse macrophage cell line (J774) was used. J774 cells were seeded in Dulbecco's Modification of Eagle's

Table 1. Bactericidal activity of peptides at 25 µM

Bacterium	LL37	WLBU2	WR12
F. tularensis	0*	98	31
B. pseudomallei	0	59	0
Y. pestis	35	81	94

*Mean per cent killing compared with control cultures, $n = 3$ repetitions.

Medium (DMEM; Cellgro) supplemented with 10% fetal bovine serum at 37 °C on 12-well cell culture plates at a density of 3×10^7 cells per well. Seeded plates were incubated overnight at 37 °C, 5% CO_2, 95% humidity. Cells were infected with *F. tularensis* SCHU S4 at an m.o.i. of 1:100 and incubated further for 2 h. Cells were washed three times with 2 ml PBS containing 50 μg gentamicin ml^{-1} followed by addition of 1 ml medium with 50 μg gentamicin ml^{-1} to each well and incubated for 1 h. Subsequently, cells were washed again three times with PBS and different concentrations of WLBU2 (3 μM to 25 μM) were added to each well and incubated for another 1 h. J774 cells were then lysed using 0.02% SDS and 100 μl of the lysate was further diluted in PBS and plated on CHA. Colonies were counted to determine bacterial count reduction with WLBU2.

Statistical analysis. Analysis was performed using percentages. Determination of significant differences in bacterial killing or inhibition between different peptides was conducted using a two-way ANOVA in Graphpad Prism 6. A $P \leq 0.001$ was considered to be statistically significant.

RESULTS

Direct killing of *F. tularensis*, *Y. pestis* and *B. pseudomallei* by eCAPs

As an initial evaluation of the test peptides, we evaluated the direct bactericidal activity of the engineered peptides against the target bacteria, as compared with the natural LL37 peptide. Both WLBU2 and WR12 outperformed LL37 against *F. tularensis* in bacterial killing (Fig. 1a). WLBU2 killed 50% of *F. tularensis* at ~2 μM while WR12 required 50 μM to achieve the same effect; LL37 only achieved 2.5% killing of *F. tularensis* at 100 μM. Two-way ANOVA revealed statistically significant differences between WLBU2 and LL37 at concentrations ≥1.5 μM and between WR12 and LL37 at concentrations ≥25 μM ($P<0.0001$). WLBU2 had significantly better antimicrobial activity against *F. tularensis* than WR12 at concentrations ≤50 μM ($P<0.0001$). At 25 μM, WLBU2 achieved 98% killing compared with only 31% killing for WR12 and less than 1% killing for LL37 (Table 1).

Initial results suggested that none of the three antimicrobial peptides tested was able to kill *B. pseudomallei* (data not shown). Another group has reported killing of *B. pseudomallei* with LL37 in the presence of potassium phosphate buffer rather than PBS (Kanthawong *et al.*, 2009, 2012). Even with potassium phosphate buffer, however, we did not observe reduced bacterial counts of *B. pseudomallei* with either LL37 or WR12, while WLBU2 achieved at best 61% killing at 100 μM (Fig. 1b). Statistical analysis indicated that the antimicrobial activity for WLBU2 against *B. pseudomallei* was significant compared with both WR12 and LL37. At 25 μM, WLBU2 achieved 59% killing, while with WR12 and LL37 no detectable killing of *B. pseudomallei* was observed (Table 1).

Both WLBU2 and WR12 were significantly more effective at killing *Y. pestis* than was LL37. WLBU2 and WR12 produced a 50% reduction in bacterial count at a peptide concentration of 1.5–2 μM, while LL37 required 100 μM to achieve 50% killing (Fig. 1c). Statistical analysis revealed a significant difference between WLBU2 and LL37 bactericidal activity at concentrations ≥3 μM and between WR12 and LL37 at concentrations ≥1.5 μM ($P<0.0001$). No significant difference

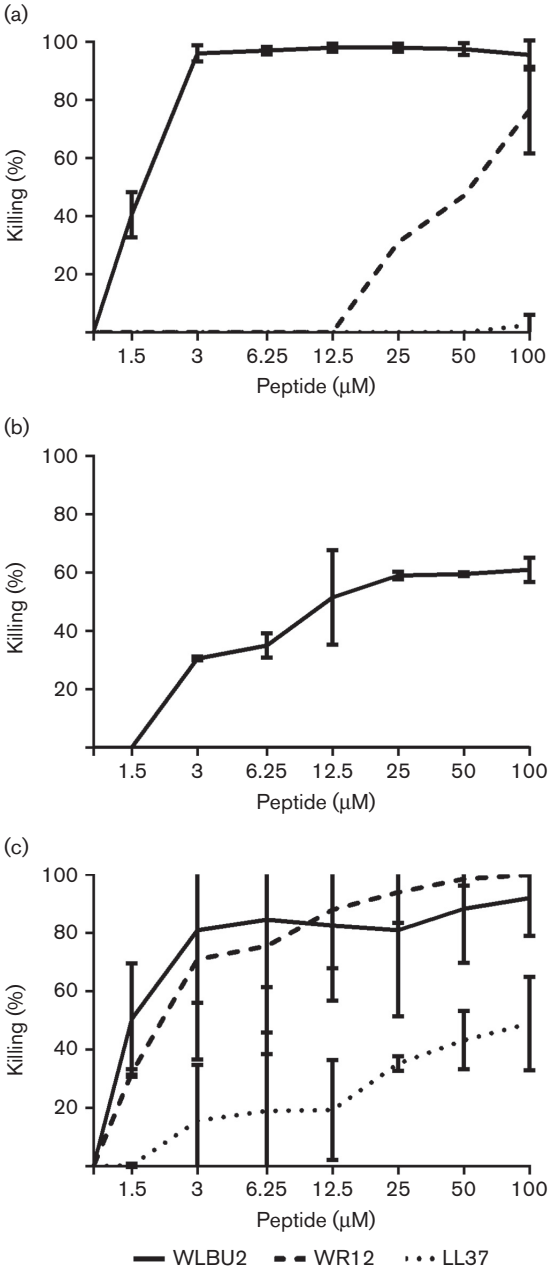

Fig. 1. Dose-dependent killing of *F. tularensis*, *B. pseudomallei* and *Y. pestis* by WLBU2, WR12 and LL37. Bacterial cultures ($1–2 \times 10^6$ c.f.u. ml^{-1}) were incubated for 1 h with twofold dilutions of the peptides diluted in PBS. Graphs show percentage reduction in bacterial count (*y*-axis) upon treatment plotted as a function of peptide concentration (*x*-axis) for *F. tularensis* (a), *B. pseudomallei* (b) and *Y. pestis* (c). The data shown are the mean of three independent experimental trials; error bars are the SD.

between WLBU2 and WR12 was found against *Y. pestis*. At 25 μM, WLBU2 achieved 81% killing, while WR12 achieved 94% killing; in contrast, LL37 achieved only 35% killing against *Y. pestis* at 25 μM (Table 1).

Growth inhibition of *F. tularensis*, *Y. pestis* and *B. pseudomallei* by eCAPs

While the preceding assays evaluated the direct killing activity of the test peptides in PBS, we next evaluated the ability of each peptide to inhibit bacterial growth in nutrient media. WLBU2 displayed the highest level of growth inhibition activity against *F. tularensis* with >90% inhibition at 25 μM (Fig. 2a). WR12 had moderate inhibitory activity at concentrations ≥12.5 μM, but only achieved 80% inhibition at the highest concentration tested (100 μM). LL37 was only slightly less capable than WLBU2 at inhibiting the growth of *F. tularensis*. By two-way ANOVA the results for WLBU2 and LL37 were not significantly different from one another. Both LL37 and WLBU2 were significantly better than WR12 at inhibiting the growth of *F. tularensis* at concentrations greater than 1.5 μM. At 25 μM, WLBU2 inhibited growth of *F. tularensis* by 92% while LL37 displayed 87% inhibition and WR12 achieved 65% inhibition (Table 2).

With *B. pseudomallei*, both WLBU2 and LL37 were able to achieve ~40% growth inhibition at 1.5 μM; growth inhibition by WR12 was lower at 1.5 μM but not significantly. Growth inhibition largely remained flat at concentrations >1.5 μM, achieving between 50 and 60% inhibition at 100 μM, the highest concentration tested (Fig. 2b). At 25 μM, WLBU2 inhibited growth of *B. pseudomallei* by 54% while LL37 produced 52% inhibition and WR12 achieved 51% inhibition (Table 2). Statistical analysis indicated no significant difference in activity between the three peptides at any of the peptide concentrations tested.

Similar to the *B. pseudomallei* results, all three peptides were able to inhibit the growth of *Y. pestis*, but only inhibited growth 50–60% at the highest concentration tested (Fig. 2c). At 25 μM, WLBU2 inhibited growth of *Y. pestis* by 44% while LL37 produced 45% inhibition and WR12 achieved 36% inhibition (Table 2). There were no significant differences between the three peptides for inhibition of *Y. pestis* at any of the peptide concentrations tested.

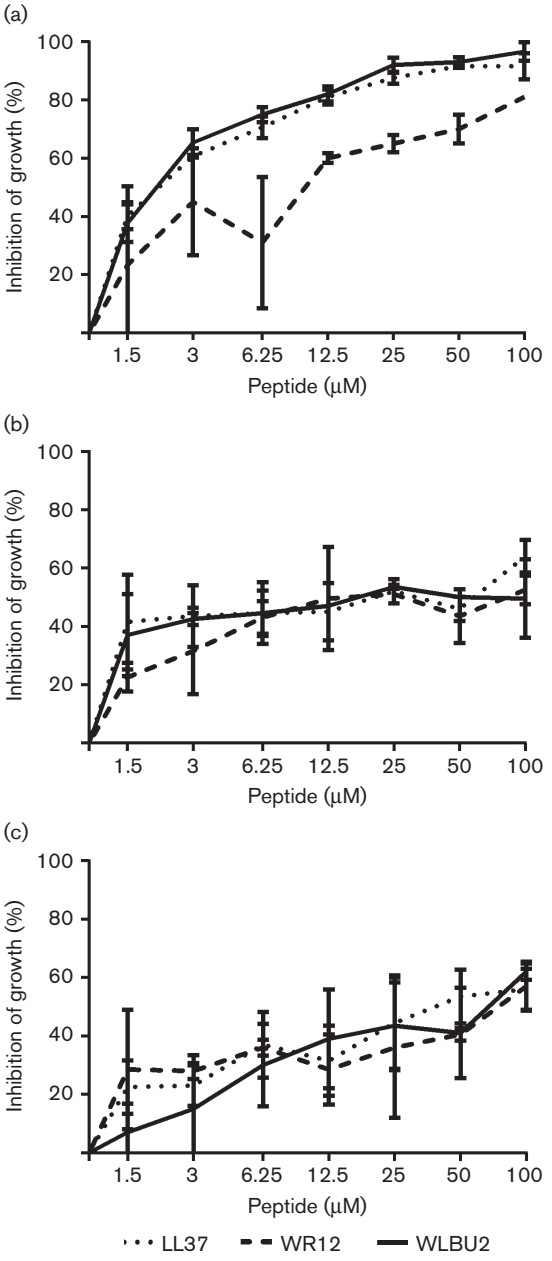

Fig. 2. Growth inhibition of *F. tularensis*, *Y. pestis* and *B. pseudomallei* by WLBU2, WR12 and LL37. Bacterial cultures ($1–2 \times 10^6$ c.f.u. ml^{-1}) were cultured overnight in BHI with twofold dilutions of the peptide. Graphs show percentage inhibition in bacterial growth (*y*-axis) upon treatment plotted as a function of peptide concentration (*x*-axis) for *F. tularensis* (a), *B. pseudomallei* (b) and *Y. pestis* (c). The data shown are the mean of three independent experimental trials; error bars are the SD.

WLBU2 can kill *F. tularensis* inside infected macrophages

In vitro tests proved that of the three peptides, WLBU2 had the highest level of antimicrobial activity against *F. tularensis*. It is well established that *F. tularensis* is a facultative intracellular bacterium that can infect a wide variety of cell types, although it is thought to preferentially infect macrophages and dendritic cells. It was therefore important to demonstrate whether antimicrobial peptides can kill *F. tularensis* inside infected eukaryotic cells. To determine the intracellular antimicrobial effect of WLBU2, we used a well-established *ex vivo* infection model wherein a mouse macrophage cell line (J774A cells) is infected with SCHU S4. SCHU S4 readily infects J774A cells and replicates in the cytosol over 24–72 h in the presence of gentamicin that kills any extracellular bacteria.

Table 2. Growth inhibition by peptides at 25 μM

Bacterium	LL37	WLBU2	WR12
F. tularensis	87*	92	65
B. pseudomallei	52	54	51
Y. pestis	45	44	36

*Averaged result, per cent inhibition compared with control cultures, $n = 3$ repetitions.

To determine the intracellular antimicrobial effect of WLBU2, *ex vivo* bacterial clearance of SCHU S4 from infected macrophage cells (J774A.1) was assessed using concentrations ranging from 3 μM to 25 μM. Fig. 3 illustrates the dose-dependent killing of intracellular *F. tularensis* by WLBU2. WLBU2 demonstrated a high antimicrobial efficacy against intracellular bacteria with only 20% bacteria remaining after 1 h of incubation with 12.5 μM and 10% at 25 μM. Thus, these data demonstrate the ability of WLBU2 to efficiently penetrate the mammalian cell membrane and effectively inactivate intracellular bacteria.

DISCUSSION

The results of the current study reveal for the first time to our knowledge the markedly increased efficacy of eCAPs against key biodefence bacterial pathogens compared with a reference natural antimicrobial peptide, LL37. The two eCAPs demonstrated superior bactericidal activity against *F. tularensis* and *Y. pestis* when compared in parallel with a natural antimicrobial peptide (LL37), thus demonstrating the improvements derived from the rational design of peptide structure. WLBU2 proved to be the best candidate against *F. tularensis* with a single dose of 3 μM peptide able to achieve a 90% reduction in bacterial count and >90% killing at 25 μM. Both WLBU2 and WR12 achieved similar results against *Y. pestis*, although only WR12 at 50 μM was able to attain >90% killing. *B. pseudomallei* provided a greater challenge with only moderate susceptibility to WLBU2. Nevertheless, WLBU2 antimicrobial effectiveness against *B. pseudomallei* surpassed that of LL37 in our hands. Finally, WLBU2 achieved 80–90% activity against intracellular *F. tularensis* in an *ex vivo* infection model. These data demonstrate the utility of eCAPs against these three bacteria, all of which cause endemic infectious diseases and are considered potential bioweapon threats.

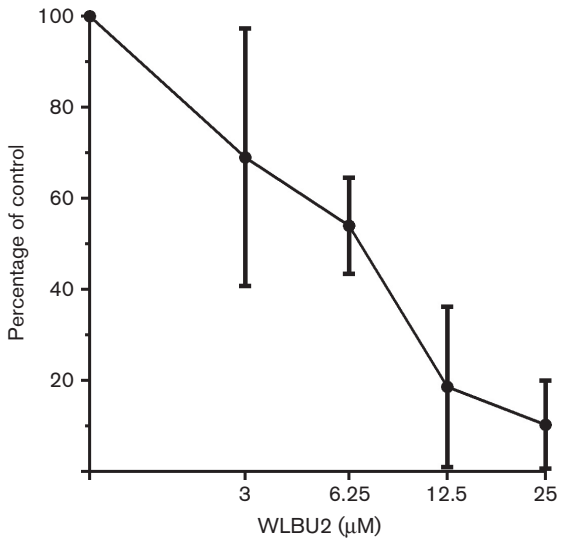

Fig. 3. WLBU2 kills *F. tularensis* inside infected macrophages. *F. tularensis*-infected J774A cells (m.o.i. 1 : 100) were incubated for 1 h with twofold dilutions of WLBU2. The percentage of bacteria remaining (*y*-axis) upon treatment is plotted as a function of peptide concentration (*x*-axis). The data shown are the mean of three independent experimental trials; error bars are the SD.

The generally poor activity of WLBU2, WR12 and LL37 against *B. pseudomallei* was not expected. Results published by another group have demonstrated bactericidal activity for LL37 and derivatives against *B. pseudomallei* (Kanthawong *et al.*, 2009, 2012). One potential difference was the choice of buffer for the bactericidal activity as we typically employ PBS, while the prior results with LL37 used potassium phosphate buffer lacking NaCl that can suppress CAP antibacterial activity of CAPs. Using potassium phosphate buffer in our assay did result in increased killing with WLBU2, but did not improve LL37 or WR12 killing. We did note that we used a higher starting concentration of bacteria (10^6 c.f.u. ml^{-1}) compared with the 10^5 c.f.u. ml^{-1} used in the previously published study (Kanthawong *et al.*, 2009, 2012). The strains of *B. pseudomallei* and choice of culture medium were also different, which may contribute to the different results obtained in the two studies. It has been noted with *F. tularensis* and *B. pseudomallei* that culture conditions alter gene and protein expression, which can impact virulence and aerosolization (Chantratita *et al.*, 2007; Hazlett *et al.*, 2008; Faith *et al.*, 2012).

The mechanism of action of eCAPs is still under investigation, but the general assumption is that their antimicrobial action stems from their ability to attach and disrupt the outer bacterial membrane leading to depolarization, metabolite leakage, and ultimately death of the bacteria (Brogden, 2005). The variability of surface-exposed structures on the outer membrane and capsule of the three pathogens in this study may explain the difference in the antimicrobial activity of the eCAPs. Several studies have verified the influence of certain antigens found on the surface of *Y. pestis* and *F. tularensis* in increasing their susceptibility to certain CAPs. For example, Galván *et al.* (2008) demonstrated that the susceptibility of *Y. pestis* to CAPs such as cathelicidin (LL37) is mediated by the capsular antigen fraction 1. Also, Vonkavaara *et al.* (2013) illustrated the importance of surface lipid A and Kdo core for interactions of *F. tularensis* with CAPs. On the other

hand, Madhongsa *et al.* (2013) demonstrated that the inhibitory activity of peptides such as polymyxin B for *B. pseudomallei* is several magnitudes higher than that against *Pseudomonas aeruginosa* or *Escherichia coli* due to the type of lipopolysaccharide (LPS) moiety on its outer membrane. Further, *B. pseudomallei* resists treatment by forming micro-colonies using its glycol-calyx polysaccharide capsule and creating a protective environment against antimicrobial peptides (Leelarasamee, 1998). This might explain the moderate antimicrobial effect of WLBU2 and failure of WR12 against *B. pseudomallei*.

Since LPS form a major part of the outer membrane of the bacteria, variation in LPS structure might contribute to the variation in bacterial susceptibility to antimicrobial peptides (Burtnick & Woods, 1999). Additional complexity on bacterial surfaces is generated by production of capsules which can be composed of polysaccharides or polypeptides. It is worth noting that *Y. pestis*, the most sensitive of the three bacteria to eCAPs in the studies reported here, has a polypeptide capsule while both *F. tularensis* and *B. pseudomallei* have polysaccharide capsules. Bacterial capsule is known to hinder complement activation and phagocytosis of *B. pseudomallei* (Reckseidler-Zenteno *et al.*, 2005). Wikraiphat *et al.* (2009) further showed that capsule mutants of *B. pseudomallei* survived poorly in macrophages and were more susceptible to antimicrobial killing by histatin and lactoferrin compared with the WT bacterial strain. Also, Hayden *et al.* (2012) suggested that genetic changes that occur in subpopulations of *B. pseudomallei* during acute infections and post-treatment are not found in the 1026b bacterial strain, which could contribute to the variance seen in the antimicrobial activity of the peptides.

F. tularensis is an intracellular organism that invades and replicates intracellularly in macrophages (Steiner *et al.*, 2014). Accordingly, for the peptides to be effective *in vivo*, they would have to exert antimicrobial activity on intracellular bacteria with minimal effect on the macrophages. WLBU2 demonstrated a high antimicrobial efficacy against intracellular bacteria with an 80% reduction in bacteria after treatment with 12.5 μM for 1 h and 90% reduction at 25 μM. These results suggest that WLBU2 has the potential to effectively treat *in vivo* infections by *F. tularensis* and other intracellular bacteria, thus increasing the activity spectrum previously reported for this lead eCAP peptide.

In this study, we demonstrated the potential of WLBU2 as a broad-spectrum antimicrobial agent against *F. tularensis* and *Y. pestis*. The results were less conclusive for *B. pseudomallei* although this may be a result of the assays or culture media employed. The results suggest that in a mass casualty setting, eCAPs might be useful in at least slowing the infection until a diagnosis can be made and appropriate treatment initiated. Further research of WLBU2, WR12 and other eCAPs against these bacteria is warranted, particularly with animal studies to evaluate the *in vivo* safety, pharmacology and efficacy of eCAPs as an antimicrobial treatment.

ACKNOWLEDGEMENTS

These studies were supported in part by funds from the Center for Vaccine Research of the University of Pittsburgh. Dr Ronald Montelaro and Dr Jonathan Steckbeck hold stock in Peptilogics. Dr Montelaro serves on an advisory board for Peptilogics, and Dr Steckbeck has a management role at Peptilogics. Although a financial conflict of interest was identified based on the authors' relationship with Peptilogics, the research findings included in this publication may not necessarily be related to the interests of Peptilogics.

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