NOVEL ENGINEERED CATIONIC ANTIMICROBIAL PEPTIDES HAVE A BROAD-SPECTRUM ACTIVITY AGAINST: FRANCISELLA TULARENSIS, BURKHOLDERIA PSEUDOMALLI AND YERSINIA PESTIS

by

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ABSTRACT

Broad spectrum antimicrobial activity against biodefense pathogens is highly desirable as a delay in treatment of infection with these pathogens can be fatal. Engineered cationic antimicrobial peptides (eCAPs) are *de novo* synthesized amphipathic agents with broad spectrum activities against gram-positive and gram-negative bacteria including *Pseudomonas aeruginosa* and methicillin-resistant *Staphylococcus aureus* (MRSA). We evaluated eCAPs designated as WLBU2 and WR12 against three bacterial pathogens that are considered potential biological weapons and a great public health concern. Using both bacterial killing and growth inhibition assays, different concentrations of WLBU2 and WR12 were tested *in vitro* against *Francisella tularensis*, *Burkholderia pseudomallei*, and *Yersinia pestis*. LL37, a natural mammalian antimicrobial peptide was used for comparison. WLBU2 proved to be the best candidate against *F. tularensis* with a single dose of 3μM needed to achieve a 90% reduction in bacterial count and a minimum inhibitory concentration (MIC) of 25 μM. LL37 failed to achieve more than 8% reduction in the bacterial count of *F. tularensis* with concentrations up to 100 μM. Both WLBU2 and WR12 achieved significantly better results against *Y. pestis* compared to LL37, but only WR12 was able to attain a MBC of 50 μM. Results for *B. pseudomallei* varied depending on the testing environment; a substantial reduction in bacterial count was seen with WLBU2 in potassium phosphate buffer but not in phosphate buffered saline. WLBU3 and WLBU4 were also tested

against *B. pseudomallei* and results indicate that increasing the length of the peptides did not enhance their activity against *B. pseudomallei*. Moreover, WLBU2 was tested against *Francisella tularensis* (SchuS4) infected macrophage cells (J774) and found to decrease intracellular bacterial count by more than 90% at a concentration of 12.5μM. This data demonstrates the potential usefulness of eCAPs against the three pathogens that were evaluated and the broad applicability of eCAPs, particularly WLBU2, against bacterial biodefense pathogens.

PREFACE

First, have a definite, clear practical ideal; a goal, an objective.

Second, have the necessary means to achieve your ends; wisdom, money, materials, and methods.

Third, adjust all your means to that end.

—Aristotle

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1.0 INTRODUCTION

Biodefense 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 agents are typically found in nature, but they could be also modified to enhance their virulence, ability to disseminate or make them resistant to current antibiotics or vaccines. Such biological agents are attractive to terrorists as an alternative to conventional weapons due to their low costs, their relative ease of production, ability to aerosolize, and difficulty of detection. Biodefense agents are classified by the National Institute of Allergy and Infectious Diseases (NIAID) according to the risk for human health into 3 categories of A, B and C [1]. In 2012, a new designation, "Tier 1", has been developed for those select agents and toxins considered to be of the greatest risk for deliberate misuse and the most significant potential for mass casualties or devastating effects on the economy or critical infrastructure [2].

Francisella tularensis, *Burkholderia pseudomallei* and *Yersinia pestis* are all gram negative facultative intracellular tier 1 select agents that are able to cause fatal diseases if not treated appropriately. The use or even threatened use of such organism can produce a widespread social disruption. Therefore, establishing effective treatment strategies against such pathogens is of the utmost importance. Unfortunately, some of these agents have already developed resistance against current available antibiotics emphasizing the need for new therapeutic agents [3][4]. In this

study, *de novo* engineered cationic antimicrobial peptides, WLBU & WR12, are investigated as a potential alternative for treatment of these organisms.

1.1 *Francisella tularensis*

Francisella tularensis is a small, pleomorphic, gram-negative coccobacillus and the causative agent of the bacterial zoonotic disease, tularemia [5]. *F. tularensis* was first isolated by McCoy in 1912 following an outbreak of a plague-like disease in rodents in Tulare County, California [6]. Since then four different subspecies of *F. tularensis* have been identified: *tularensis*, *holarctica*, *mediasiatica and novicida* [7]. Most human cases are caused by either subspecies *holarctica* or *tularensis*, with the latter being the more pathogenic subspecies. With the exception of a presumptive laboratory release, subspecies *tularensis* is only found in North America.

Since its identification, tularemia cases have been documented throughout the Northern hemisphere including Europe, China, Korea, Japan, the former Soviet Union and North America. Up until the 1950s, the United States had thousands of tularemia cases reported annually with a peak number of 2,291 in 1939 [8]. With the discovery of antibiotics these numbers declined drastically reaching less than 150 cases per year between 1990 and 2000. Except for Hawaii, all American states have reported at least one case of tularemia with the majority of them residing in the south-central and western region (Arkansas, Missouri, South Dakota, and Oklahoma) [9].

Francisella tularensis is thought to be maintained in soil, water and through its extensive host distribution including humans, domesticated and wild animals such as lagomorphs, carnivores, rodents, birds and fish [10, 11]. It is primarily transmitted to humans and other animals through arthropod vectors such as biting flies and ticks. Ninety percent of tularemia cases in the United States occur via an arthropod bite, more specifically via the American dog tick, the Lone

Star tick, and the Rocky Mountain wood tick [10, 12]. It can also occur directly through contaminated food, water, infected animals and their carcasses [13]. Aerosolization of *F. tularensis* can occur during handling of contaminated material which may lead to the inhalation of the bacteria. For instance, 11 cases of primary pneumonic tularemia occurred in the summer of 2000 on Martha's Vineyard Massachusetts due to the aerosolization of the bacteria from infected animal carcasses and their dens during lawn mowing [14]. Further, laboratory transmission of *F. tularensis* can occur readily due to the ease of its aerosolization; opening a culture plate is enough to create an aerosol [15]. To date, there has been no supportive evidence of person to person transmission.

The onset of tularemia is usually abrupt with flu-like symptoms, fever and generalized weakness. Chest tightness, sub-sternal pain and dry cough have also been noted [16]. Following an incubation period between 1 to 21 days and depending on the route of inoculation, tularemia manifests in one of six forms: oropharyngeal, pneumonic, typhoidal, ulceroglandular, glandular or oculoglandular [17]. The most common form of the disease is ulceroglandular tularemia following an infected arthropod bite or handling contaminated animals [18]. Pneumonic tularemia results from inhaling contaminated aerosols or through the dissemination of the bacteria from another site. It is characterized by an acute illness with pharyngitis, pleuropneumonitis, bronchiolitis and/or hilar lymphadenitis [19]. Patients may progress into respiratory failure and death. Typhoidal tularemia has no specific site of inoculation and is characterized with systemic illness including fever, abdominal pain, and diarrhea and vomiting. If not treated promptly, both typhoidal and pneumonic tularemia may lead to death [13].

In situations of contained tularemia causalities, where individual patient management is possible, a parenteral route of treatment with antibiotics is recommended. The aminoglycosides

streptomycin and gentamicin are both bactericidal against *F. tularensis* and are considered the gold standard for treatment in these situations. Streptomycin (1 g IM twice daily) or gentamicin (5 mg/kg IM or IV once daily) should be continued for 10 days. Tetracycline or chloramphenicol are bacteriostatic against *F. tularensis* and can be used as alternative treatment. Due to their bacteriostatic profile, these drugs should be continued up to 14 days to reduce the possibility of relapse [13](23).

Fluoroquinolones, such as ciprofloxacin and doxycycline, have a bactericidal effect against intracellular organisms such as *F. tularensis*. During a human outbreak in Spain (between December 1997 and February 1998) ciprofloxacin treatment resulted in the lowest therapeutic failure and side effects compared to streptomycin and doxycycline [20]. Currently, ciprofloxacin can be regarded as the drug of choice for the treatment of uncomplicated tularemia and as a second-line treatment for severe infections; although relapse may occur due to incomplete clearance of *F. tularensis*. In case of massive causalities, e.g. during a bioterrorism attack, oral doxycycline and ciprofloxacin are the preferred choices for both adults and children. Achieving the appropriate treatment decreases the mortality rate of tularemia from 35% to 1% [21]. Post-exposure prophylactic treatment would include streptomycin, gentamicin, doxycycline, or ciprofloxacin for 14 days [13]. Even with antibiotics available for treatment of *F. tularensis* infection, pneumonic tularemia requires intensive care and the recovery rate is extremely slow so that even a moderate outbreak can overwhelm medical facilities. Further, *F. tularensis* can be genetically modified to become antibiotic resistant.

The *F. tularensis* live vaccine strain (LVS) is the only vaccine available to protect against tularemia. LVS was generated in the former Soviet Union as a classically passaged live attenuated strain derived from a virulent *F. tularensis subsp. holarctica* strain. Studies conducted in the

1960's demonstrated that LVS was safe when given to humans and could protect against aerosol challenge with SchuS4, a virulent strain of *F. tularensis subsp. tularensis* although this protection failed at higher challenge doses. Nevertheless, LVS is only available as an Investigational New Drug (IND) and unlikely to be approved by the FDA due to concerns regarding the potential for reversion and incomplete protection against aerosolized *F. tularensis*. As an IND, LVS is currently given to at-risk laboratory personnel who work with tularemia. Consequently, there is no actual way of controlling this disease in nature. Public awareness of this organism and its potential harm to humans should be a priority. In regions where tularemia is endemic, people should avoid handling sick or dead animals and insect populations should be reduced [22].

F. tularensis is considered one of the most infectious bacterial pathogens. In a bioterrorist attack *F. tularensis* would most likely be used as an aerosol resulting in pneumonic tularemia represented as respiratory symptoms, sub-sternal discomfort, and cough. In 1970, a World Health Organization (WHO) committee stated that a 50-kg aerosol dispersion of a virulent *F. tularensis* strain in a population area of five million people would cause 19,000 deaths and 250,000 hospitalizations. Without the appropriate antibiotics, most of the hospitalized cases would progress into respiratory failure and death [23]. *F. tularensis* progression is slower than anthrax or plague, but the disease caused by this organism persists for several weeks with a high relapse rate. The WHO estimated that such an attack with tularemia would cost around $5.4 billion per 100,000 persons infected [24].

1.2 *Burkholderia pseudomallei*

Burkholderia pseudomallei is a gram-negative, motile rod-shaped, soil saprophyte that can be easily collected from water and wet soil in endemic areas. *B. pseudomallei* is the causative agent of melioidosis, a tropical infectious disease affecting humans, animals and plants. Previously known as *Pseudomonas pseudomallei*, *B. pseudomallei* is able to infect various organs causing a range of clinical symptoms that might be fatal to the infected patient. Despite its rare presence in the western countries, concern about the potential use of *B. pseudomallei* in bioterrorism fuels a great deal of the *B. pseudomallei* research.

Melioidosis is endemic in various areas of Southeast Asian and northern Australia. Thailand has the highest annual incidence of melioidosis; 4.4 cases per 100,000. In Ubon Ratchathani – Thailand, 20% of community acquired bacterial infections are due to *B. pseudomallei* [25]. Further, severe melioidosis has a case fatality rate of 50% in that region [26]. Northern Australia has about the same incidence rate as Thailand, and *B. pseudomallei* is considered the most common case for fatal community acquired bacterial pneumonia; the mortality rate is about 20% among patients with melioidosis [27]. Sporadic cases were observed worldwide, particularly in the Caribbean region, Africa and the Middle East [28]. Cases that occurred in the western countries usually involved returning travelers and military veterans [29].

Humans and animals can be infected through ingestion, inhalation or inoculation of a skin wound. In humans, most cases occur directly from the environment through skin abrasions. Inhalation of *B. pseudomallei* is usually related to heavy rainfalls and strong wind that might carry it from the soil or water into our respiratory system [30]. Person to person transmission is rare, but has been reported between family members in close contact [31]. Vertical transmission is also

considered rare with only a few newborn cases reported [32]. Transmission by mosquitoes and rat fleas has been reported, but the role of vector born transmission remains unclear [28, 33].

Melioidosis is often referred to as "the great imitator" due to its wide spectrum of clinical diseases. Melioidosis can present as an acute or chronic, localized or generalized disease. Other cases may result in an inapparent infection with no noticeable signs or symptoms [34]. Following an incubation period between 1 and 21 days, the lung is mostly affected by this organism. The ensuing pulmonary disease can range from mild respiratory illness to acute or chronic pneumonia that may rapidly progress to septic shock and death. Pneumonia is the most common manifestation between all patients with melioidosis [25] and may progress to septicemia if left untreated. Septicemic melioidosis is the most serious form of the disease with a mortality rate as high as 70% [35][36].

Despite being intrinsically resistant to many drugs, *B. pseudomallei* is susceptible to some antibiotics. Treatment is divided into 2 phases: intensive and eradication. As a general rule, intravenous bactericidal drugs are used in the intensive phase, while oral bacteriostatic drugs are used in the eradication phase. In life threatening disease, the current regimen is comprised of intravenous Ceftazidime for at least 2 weeks followed by oral combination of ciprofloxacin and azithromycin or doxycycline and co-trimoxazole for 20 weeks to prevent relapse. For mild to moderate disease, IV amoxycillin-clavulanate is used for 2 weeks followed by co-trimoxazole and doxycycline for 20 weeks. Localized melioidosis should be treated with incision and drainage with co- trimoxazole for 6 – 8 weeks [37].

Relapse and recurrence of infection is common in melioidosis despite receiving appropriate therapy. Relapse is defined as "reappearance of signs and symptoms after clinical response while still on antimicrobial therapy" while recurrent infection is "a new episode of melioidosis caused

by the same organism after full clinical recovery" [38]. Both infections are thought to be due to failure of the host to eliminate the offending pathogen during initial attack. Many factors aid *B. pseudomallei* evasion of the host system including hiding in phagocytic cells and sealed abscesses. In endemic areas such as Malaysia and northeast Thailand the overall relapse rate is higher than 15% per year despite prolonged treatment [39].

With no effective vaccine available, precautionary measures should be taken in endemic areas. These measures include covering all open wounds and wearing the appropriate gear for outdoor activities. Infected animals are usually euthanized rather than treated due to the high risk of relapse and expensive medication needed. If treated, animals should be under lifelong monitoring for relapse [38].

CDC assigned *B. pseudomallei* as a category B select agent and a possible bioweapon leading to an increased research interest around the world. Even in literature, this agent's potential as a bioweapon was suggested by Sir Arthur Conan-Doyle in his Sherlock Holmes story "The Dying Detective" [40]. It is believed that *B. pseudomallei* was being weaponized by the former USSR with the potential of creating an engineered antibiotic resistant strain. Although the prospective of weaponizing this agent is uncertain, it has a great potential due to its high mortality rates, wide range of hosts, ability to be aerosolized and intrinsic antibiotic resistance

1.3 *Yersinia pestis*

Yersinia pestis is a gram-negative, non-motile, non-spore forming coccobacillus belonging to the family Enterobacteriaceae. *Y. pestis* is believed to be a clone of *Y. pseudotuberculosis* that emerged within the last 1,500 to 20,000 years by acquiring different genetic elements making it able to be transmitted through fleas [41]. *Y. pestis* had numerous nomenclature changes from

Bacterium pestis to *Bacillus pestis* to *Pasteurella pestis* and finally *Y. pestis* in 1970 [42]. Plague (a.k.a the Black Death) is the zoonotic disease caused by this organism. It can be transmitted to humans through flea bites causing a rapidly progressive severe illness that is usually deadly if not appropriately treated.

Plague was the cause of three major pandemics in which nearly 200 million people were killed. The first pandemic, the Justinian plague, started in Egypt around A.D. 541 and then swiftly transmitted through the Middle East and Mediterranean Europe leading to the loss of 50 – 60% of the population. The second pandemic started with an epidemic in Europe during the 14th century. This epidemic killed 17 to 28 million Europeans (30 – 40% of the population) and more importantly resulted in relentless epidemic cycles that comprise the second pandemic. The third pandemic started in China around 1855 and disseminated to Africa, Australia, Europe, Middle East, North America, and South America by 1900. It is believed that plague was introduced to the United States from China in the 20th through ship rats and their fleas. Fortunately, mortality rates were significantly reduced compared to previous pandemics which is thought to be due to effective antibiotics and active public health measures [43]. Currently, it is believed that around 4,000 human plague cases occur annually worldwide [44].

Plague is enzootic in rodents, but it can be transmitted to humans by infected fleas. When the rat fleas, the classical vector for plague, feed on an infected animal the bacteria infects and multiplies in the midgut of the flea. Ultimately the flea dies out of starvation, but not before infecting several hosts. In the infected mammal, *Y. pestis* spreads from the bite site to the regional lymph node and multiply quickly causing the lymph node to swell and form a bubo. Further, the bacteria spread to the bloodstream and colonize in different organs such as liver, spleen and lung [45].

Y. pestis can rarely transmit to humans through direct contact with infected animals especially rodents. Person-to-person transmission through respiratory droplets may occur; in 1994 an outbreak of pneumonic plague occurred in Surat, India. A total of 460 plague cases were compiled between September 20 and September 25 in Surat. When news of the plague became known to the public about 700,000 people fled the city. It is estimated that this small outbreak cost India US$3 billion and the global economy US$5 to $6 billion [46]. Fortunately pneumonic plague is rare, but bubonic plague may spread to the lungs triggering a secondary pneumonic plague that can be transmitted to other people and cause considerable mortality if untreated [43].

Plague manifests as one of three primary forms: Bubonic, septicemic, or pneumonic. Human epidemics commonly start as bubonic plague following a flea's bite. If not treated promptly infected patients usually develop secondary plague septicemia or pneumonia increasing the risk of person to person transmission. Untreated bubonic plague may lead to 40 – 60% case fatalities while untreated septicemic and pneumonic plague is always fatal; death is usually due to a septic shock [47, 48].

Pneumonic plague is very rare, nonetheless it is the most dangerous and deadly form of the disease. Disease spreads via respiratory droplets from infected animals or humans. It initially presents as a febrile like illness but progresses rapidly with 1 to 3 days into an overwhelming pneumonia with productive bloody cough, dyspnea and cyanosis. Cervical lymph nodes may be affected creating buboes in the neck region. The disease quickly overwhelms the lungs and hemorrhages develop resulting in hemorrhagic pneumonia. The terminal events may include respiratory failure and circulatory collapse. Septicemic plague patients may develop secondary plague pneumonia through the hematogenous dissemination of the bacteria to the lung (68)[49].

Rapid diagnosis and prompt administration of appropriate antibiotics are essential for the success of treatment and reduction of complications. Streptomycin, tetracycline, and sulfonamides are considered the standard treatment, with streptomycin being the treatment of choice for plague. Using one or a combination of antibiotics depends on the severity of the infection. Gentamicin alone or with tetracycline can be used as a substitute for streptomycin. Antibiotic treatment continues for 10 days or 2 days after the fever subsides. Patients suspected of having pneumonic plague or secondary pneumonia following bubonic plague should be isolated for at least 4 days after starting treatment to prevent human-to-human transmission [50, 51]. A recent study by Layton et al demonstrated successful treatment of established pneumonic plague using Levofloxacin in African green monkeys. The monkeys were given a respiratory dose of 100 lethal doses for 50% of animals (LD50) and were observed for fever and vital signs by telemetry. Intravenous Levofloxacin treatment was initiated when the animal spiked a fever in a fashion that mimics antibiotic levels achieved in humans. All animals treated with a placebo died, while all animals treated for 10 days with Levofloxacin survived. In the treated group fever resolved within 24–48 hours of initiating treatment. This study indicates that Levofloxacin may be a suitable broad-spectrum antibiotic for therapy in an aerosolized bioweapons attack and should be further studied and evaluated for bubonic plague [52].

Prophylactic therapy consists of sulfonamide, trimethoprim-sulfamethoxazole, or tetracycline administered to the patient and their close contacts. In the case of a bioterrorist attack with aerosolized plague, streptomycin or gentamicin are the preferred choice in a contained casualty setting, while oral therapy with doxycycline, tetracycline, Levofloxacin or ciprofloxacin are preferred for mass casualty situations. There are two plague vaccines currently available around the world: a live vaccine made from an attenuated strain of *Y. pestis* and a killed vaccine

derived from a formalin-fixed virulent strain. Vaccines are administered intramuscularly as a series of three primary shots over a period of a year then booster shots should be given every 1 to 2 years. Due to their side effects, need for repeated boosters and ineffectiveness against pneumonic plague, these vaccines are only given to people at high risk of contracting *Y. pestis* such as military personnel serving in endemic areas [53, 54].

In 1995, two multi-drug resistant isolates of *Y. pestis* were observed in Madagascar. One strain was isolated from a 16 year old male and the other from a 14 year old patient with both of them presenting symptoms of bubonic plague. Both strains were resistant to streptomycin and several other antibiotics recommended for plague treatment. Both patients were successfully treated with a combination of intramuscular injections of streptomycin (2 g per day for 4 days) and oral administration of trimethoprim-sulfamethoxazole (2 g per day for 10 days) [50]. Thus there is cause for concern that antibiotic resistant strains of *Y. pestis* could occur naturally or be made in a laboratory.

Due to its high fatality and rapid progression, *Y. pestis* is considered a potential biological weapon. During the World War II, the Japanese army released plague-infected fleas over several areas in China leading to many plague outbreaks [55]. Offensive biological weapons programs in both the USA and the Soviet Union researched and successfully developed techniques to aerosolize plague in place of using infected fleas. It was estimated by the WHO that releasing an aerosol of 50kg of *Y. pestis* over a city of 5 million would lead to development of pneumonic plague in 150,000 people, of which 36,000 are expected to die [56]. Research in weaponizing *Y. pestis* was terminated in the USA around 1970 (when all offensive biological warfare research was terminated) but it continued in the Soviet Union. In the year 1995 an individual in Ohio was arrested after illegally acquiring *Y. pestis* by mail. This incident lead to establishment of new

antiterrorism regulations which included regulations guiding tracking shipments (and eventually inventories) of pathogens designated as ‘select agents’, those for which there was concern they could be used as biological weapons. Today *Y. pestis* is classified as a tier 1 select agent by the United States Department of Health and Human Services [57].

1.4 Engineered Cationic Antimicrobial Peptides (eCAPs)

Cationic antimicrobial peptides (CAPs) are amphipathic peptides of 12–50 amino acids 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 [58]. In response to infectious pathogens, CAPs can be released from macrophages, granules of neutrophils, or with mucosal and skin secretions from epithelial cells. CAPs can act against a wide range of targets including gram-positive and gram-negative bacteria, fungi and parasites - the only essential component in these targets is a negatively charged plasma membrane. Therefore, normal cells have a relative resistance due to the peptides preference for negatively charged, cholesterol free membranes [59]. The mechanism of action of CAPs is not fully understood, although studies have implicated the electrostatic interaction between the peptides and the lipid molecules on the bacterial membrane. When compared to other antibiotics, CAPs are able to kill bacteria rapidly, within 30 to 180 seconds, limiting the bacterium’s ability to develop resistance against these peptides. Therefore, CAPs are considered a good candidate for use against multi-drug resistance (MDR) bacteria.

Although these cationic peptides have a wide spectrum of action against pathogens, their activity strongly depends on the environment they are released in. The optimal potency of each peptide is only achieved in specific environmental conditions and readily lost when subjected to a

different setting. For instance, human β-defensin loses its antimicrobial activity when subjected to the abnormally high NaCl concentration in a cystic fibrosis lung [60]. Therefore, to be able to utilize their antimicrobial activity to the fullest, development of CAPs has shifted towards modifying host-derived peptides or choosing specific amino acids for *de novo* synthesis. Studies of an HIV-1 transmembrane protein (referred to as lentivirus lytic peptide 1 (LLP1)) demonstrated that it had remarkable antimicrobial properties [61]. As a result of that work, it was concluded that *de novo* synthesis of an α-helical amphipathic cationic antimicrobial peptide, using arginine residues on the polar side and valine residues on the non-polar side, would greatly improve the potency and selectivity of the peptide [62]. Further studies of the influence of length and helical structure on activity lead to development of a series of *de novo* synthesized peptide designated WLBU [63]. WLBU peptides are composed of arginine in the hydrophilic face, valine and tryptophan residues in the hydrophobic face separated from each other by at least seven amino acids (Figure 1). Moreover, WLBU studies demonstrated that tryptophan substitution significantly increased antimicrobial activity in challenging environments such as serum and blood. Therefore, WR12 was designed using only arginine in the hydrophilic face and tryptophan in the hydrophobic face [63, 64] (Figure 1).

Several studies tested the potency of engineered peptide derivatives against various agents such as *Pseudomonas aeruginosa*, methicillin resistant *Staphylococcus aureus*, *Streptococcus gordonii*, *Fusobacterium nucleatum*, and *Porphyromonas gingivalis* [65-68]. Unlike the natural peptide LL37, WLBU2 was able to eliminate *P. aeruginosa* and *S. aureus* in physiological NaCL concentrations and kill *P. aeruginosa* in human serum, whole blood and skin fibroblasts without evident adverse effects to the host cells. *In vivo* toxicity and efficacy of WLBU2 against *P. aeruginosa* were also tested using a mouse model. An intravenous dose of 3mg/kg of WLBU2 was

able to clear the bacteria and save all the 14 mice from the *P. aeruginosa* infection. WLBU2 had no apparent toxic effect on the mice at up to 12 mg per kg of their body weight [64]. Further, WR12 and WLBU2 demonstrated an optimal antibacterial selectivity against a panel of MDR clinical isolates such as *Acinetobacter baumannii* and *Klebsiella pneumoniae* [66]. Taking all this into consideration, this study aims to evaluate the *in vitro* and *ex vivo* antimicrobial activity of WLBU series and WR12 against *F. tularensis, B. pseudomallei* and *Y. pestis.*

2.0 SPECIFIC AIMS

This study aims to investigate the potential role of WLBU and WR12 as a antimicrobial agents against *F. tularensis, B. pseudomallei* and *Y. pestis*. The potency of WLBU and WR12 is compared to that of LL37 under defined test condition to demonstrate the ability of *de novo* synthesized peptides with optimized amphipathic structures to overcome certain limitations of host antimicrobial.

2.1 AIM 1: Evaluate the antimicrobial activity of WLBU and WR12 *in vitro* against virulent isolates of all three bacterial pathogens (*F.tularensis, B. pseudomallei, and Y. pestis*) to establish their ability to kill or inhibit growth of the pathogens. All procedures will be first evaluated with the attenuated LVS strain of *F. tularensis* prior to use in BSL-3.

a. Determine the growth of each pathogen in specified media and the exponential phase by establishing a growth curve for each of them. This will further help in determining the equations needed for bacterial concentration calculations.

b. Determine the effectiveness of our peptides in reducing the bacterial count of the tested pathogens and the minimum bactericidal concentration (MBC), if applicable.

c. Determine the effectiveness of our peptides in inhibiting the growth of the tested pathogens and the minimum inhibitory concentration (MIC), if applicable.

2.2 AIM 2: Assess the antimicrobial activity of WLBU2 *ex vivo* with *F. tularensis* infected macrophages.

a. Determine ability of WLBU2 to modulate macrophage cells (J774) cell viability.

b. Determine the reduction in bacterial counts after using WLBU2 against *F. tularensis* (SchuS4) infected J774 cells.

3.0 METHODS AND MATERIALS

3.1 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 (PAPRs) were worn for respiratory protection, and all work was conducted in a class II biosafety cabinet using Vesphene IIse (diluted 1:128, Steris Corporation, cat. #646101) as a disinfectant.

3.2 Bacteria

All the following bacteria were obtained from Biodefense and Emerging Infections Research Resources Repository, Manassas, VA. *Francisella tularensis* live attenuated vaccine strain (LVS) and SchuS4 strain (Catalog No. NR-643) were grown on cysteine heart agar (CHA) for 2 days at 37°C and cultured in brain heart infusion broth (BHI) or Chamberlain's chemically defined medium (CCDM) for 17 to 19 hours [69]. *Burkholderia pseudomallei* strain 1026b (Catalog No. NR-9910) was grown on Luria Broth (LB) Agar for one day at 37°C and then cultured in BHI for 11 hours. *Yersinia pestis* strain CO92 (Catalog No. NR-641) was grown on tryptic soy agar (TSA) for 2 days at 37°C and then cultured in BHI or Bacto nutrient broth for 24 to 27 hours.

The overnight culture of bacteria was prepared by streaking an agar plate with a loopful of the frozen bacterial stock (-80 freezer). The plate was incubate for 24hrs at 37°C for *B. pseudomallei*, 48 hrs at 37°C for *F. tularensis* and 48hrs at 30°C for *Y. pestis*. Next, several colonies were taken from the plate and re-suspend in 3ml of growth media e.g. BHI. Optical density (OD) was adjusted to 0.1 then 0.5ml of the previous solution was added to 24.5ml of the growth media in a 125ml

filtered top flask. Bacterial culture was incubated at 37°C in a shacking incubator (250 rpm) for the number of hours needed for the bacteria to reach the exponential phase; *B. pseudomallei* (11hours), *Y. pestis* (24 – 27 hours), and *F. tularensis* (17 – 19 hours). After incubation, 10ml of the culture were spun down at 2800 rpm for 10 minutes then re-suspend in PBS or Growth media depending on the assay done.

3.3 Peptides

Peptides were synthesized using standard Fmoc (9-fluorenylmethoxycarbonyl chloride) synthesis protocols (Genscript, Piscataway, NJ). Synthetic peptides were characterized and purified by reverse-phase high pressure liquid chromatography on Vydac C18 or C4 columns (The Separations Group, Hesperia, Cal.). The identification of each was established by mass spectrometry (Electrospray Quatro II triple quadruple mass spectrometer; Mi-cromass, Inc., Manchester, United Kingdom). Peptide concentrations were determined using a quantitative ninhydrin assay. The peptides and their sequences were as follows.

WLBU2: 24-mer peptide composed of 13 arginine (R) in the hydrophilic face and 8 valine (V) and 3 tryptophan (W) residues in the hydrophobic (Figure 1)

(RRWVR RVRRV WRRVV RVVRR WVRR)

WLBU3: 36-mer peptide composed of 18 arginine in the hydrophilic face and 14 valine and 4 tryptophan residues in the hydrophobic

(VRRVW RRVVR VVRR WVRR VRRV WRRV VRVV RRWVRR)

WLBU4: 48-mer peptide composed of 23 arginine in the hydrophilic face and 20 valine and 5 tryptophan residues in the hydrophobic

(RVVRV VRRWV RRVRR VWRRV VRVVR RWVRR VRRVW RRVVR VVRRW RVV)

WR12: 12-mer peptide composed of 6 arginine in the hydrophilic face and 6 tryptophan residues in the hydrophobic; requires mannitol to prevent precipitation (Figure 1)

(RWWRWW RRWWRR)

LL37: 37-mer peptide (Leu,Gly,Asp,Phe,Arg,Lys,Ser,Glu,Ile,Val,Gln,Pro,Thr); first natural amphipathic alpha helical peptide isolated from human

(Leu - Leu - Gly - Asp - Phe - Phe - Arg - Lys - Ser - Lys - Glu - Lys - Ile - Gly - Lys - Glu - Phe - Lys - Arg - Ile - Val - Gln - Arg - Ile - Lys - Asp - Phe - Leu - Arg - Asn - Leu - Val - Pro - Arg - Thr - Glu – Ser)

3.4 Assays

3.4.1 *In vitro* assays:

Prior to any experiments, growth curves were established for each pathogen to determine log-phase and stationary phase. Also, mathematical equations were derived from calibration curves (optical density/colony-forming unit (c.f.u) curves) to achieve the needed bacterial concentration.

Bacterial killing assay (BKA): A of bacterial suspensions (concentration of 1×10^6 c.f.u/mL) in phosphate buffer saline or potassium phosphate buffer were incubated with two-fold dilutions of peptides (concentration of 1.5µM to 100µM) in a 96-well plate (Falcon No. 3072, Becton-Dickinson, Oxnard, CA) for 60 min at 37°C. Serial peptide dilutions were performed and plated on agar plates. Colonies were counted following an appropriate incubation time to determine the reduction in bacterial count. The minimum bactericidal concentration (MBC) is determined as peptide concentration reducing bacterial count by at least 99%.

Growth inhibition assay (GIA): Bacterial suspensions (concentration of 1×10^6 c.f.u/mL) in growth media were incubated with two-fold dilutions of peptides (concentration of 1.5µM to 100µM) in a 96 well plate for 24 to 48 hours at 37°C. After incubation the optical density of each well was measured at 600nm using a spectrophotometer. Percent inhibition was calculated from the difference in optical density measured. The minimum inhibitory concentration (MIC) was determined as peptide concentration reducing bacterial growth by at least 90%.

3.4.2 MTS assay:

Macrophage J774 cells (ATCC TIB – 67) were seeded in Dulbecco's Modification of Eagle's Medium (DMEM, Cellgro) supplemented with 10% fetal bovine serum at 37°C on 96-well cell culture plates at a density of 3×10^8 cells/well. A set of wells is prepared with medium only for background subtraction and another with untreated cells for 100% viability reference. Seeded Plate was incubated overnight at 37 °C, 5% CO2, 95% humidity. WLBU2 was added at concentration ranging from 3 µM to 100 µM and incubated for an hour. Following incubation cells were washed using PBS and 100 µL of media were added to each well. Then 20 µl of MTS solution, 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (Sigma, Lakehood, N.J.), were added to each well (final concentration of MTS will be 0.33 mg/ml) and incubated for 2hr at 37°C. Absorbance was recorded at 490 nm to determine the viability of cells with different peptide concentrations compared to untreated cells.

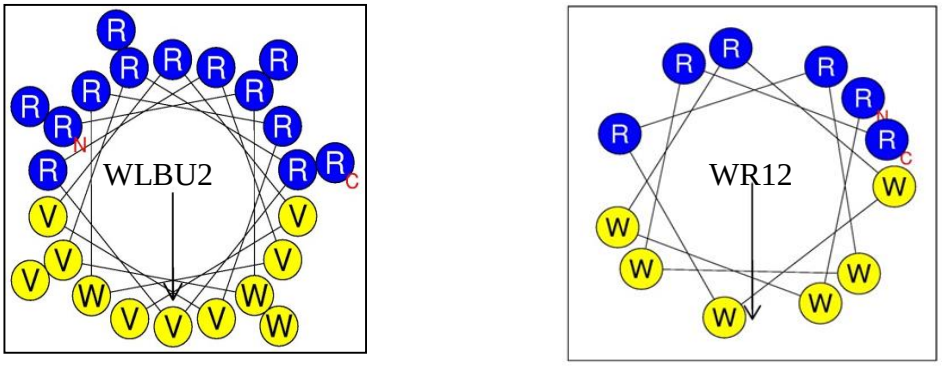
3.4.3 *Ex vivo* assay:

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 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/well. Seeded plates were incubated overnight at 37 °C, 5% CO2, 95% humidity. Cells were infected with *F. tularensis* SchuS4 at an MOI of 1:100 and incubated further for 2 hr. Cells were washed three times with 2 ml of potassium buffer saline(PBS) containing 50µg/ml gentamicin followed by addition of 1ml of media with 50µg/ml gentamicin to each well and incubated for 1 hr. Subsequently, cells were washed again three times with PBS and different concentrations of WLBU2 (3µM to 25µM) was added to each well and incubated for

another 1hr. Cells were then lysed using 0.02% sodium dodecyl sulfate (SDS) and 100µl of the lysate was further diluted in PBS and plated on CHA. Colonies were counted to determine bacterial count reduction with WLBU2.

3.5 Statistical Analysis:

Analysis was performed using percentages. Significance of differences in bacterial killing or inhibition between different peptides was conducted using two way analysis of variance (ANOVA) in Graphpad prism 6. $P < 0.0001$ was considered to be statistically significant.



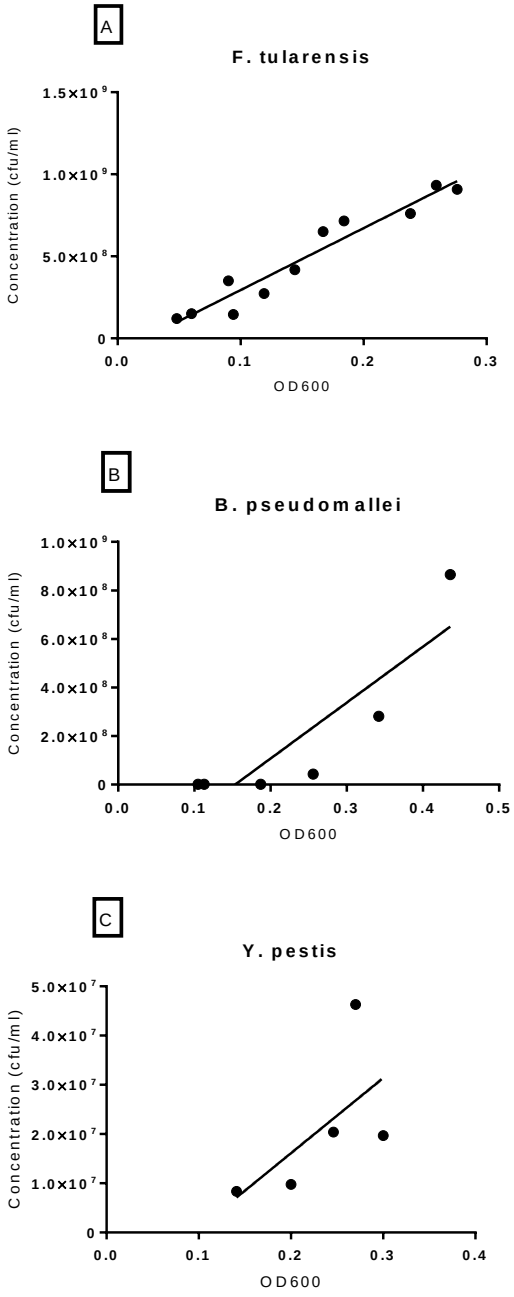
The cationic amphipathic peptides were designed as demonstrated in helical wheel diagrams. Arginine, valine, and tryptophan residues were arranged to form α helices, with the hydrophilic and hydrophobic faces indicated in blue and yellow circles, respectively. The 12- and 48-mers LBU1 and WLBU4 are shown as representatives of the LBU and WLBU series, respectively.

Figure 1 Schematics of WLBU2 and WR12 .[66]

4.0 RESULTS

4.1 Defining bacterial concentration by a standard equation for each pathogen

Light absorbance at different dilutions of bacterial cultures were measured to produce a calibration curve which enabled conversion of the optical density (OD) measured into cell counts and provided a standard equation for calculating the required concentration. This equation was obtained by growing bacteria to the exponential phase as described above, taking OD readings of several dilutions, and plating them. Concentration (measured as c.f.u/ml) was plotted against the corresponding OD (Figure 2); by performing a linear regression analysis we were able to obtain the specific equation for each bacteria (Table 1).



(a) *F. tularensis* (b) *B. pseudomallei* (c) *Y. pestis*. Each OD reading was done in triplicate. A linear regression analysis was done using data points collected for each pathogen.

Figure 2 Calibration curve to calculate the conversion factor between OD reading at 600nm and the number of colony forming units growing per ml (c.f.u/ml).

Table 1 Equations generated from the calibration curve of each bacteria.

Bacteria	**Equation**
F. tularensis	Y = 3.774e+009*X - 8.304e+007
B. Pseudomallei	Y = 2.303e+009*X - 3.535e+008
Y. pestis	Y = 8.391e+007*X - 4.056e+006

Y= bacterial concentration (c.f.u/ml), X = OD

4.2 *In vitro* antimicrobial activity of eCAPs

In order to investigate the antimicrobial activity of WLBU2 and WR12 compared to LL37, pathogens were treated with each peptide and evaluated using both a growth inhibition assay and a bacterial killing assay.

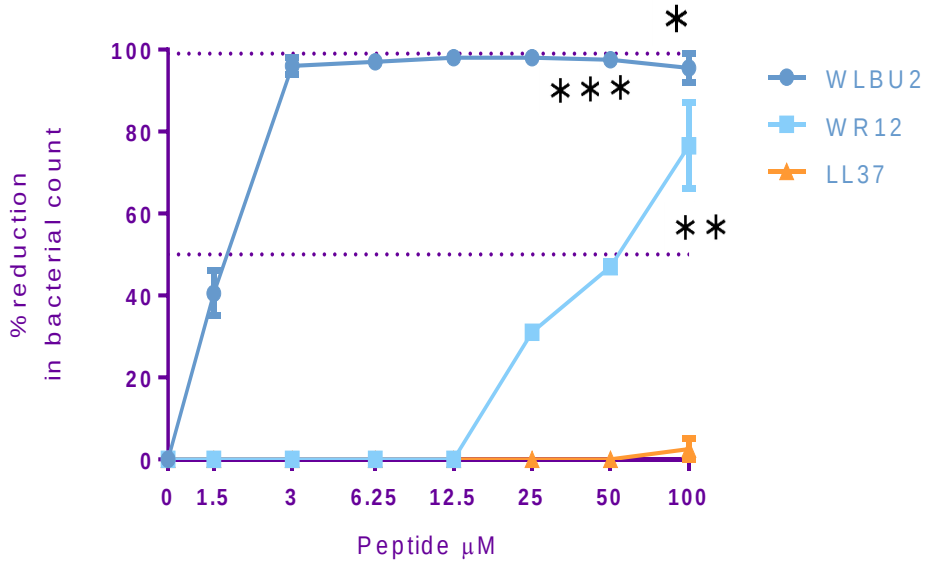
4.2.1 Bacterial Killing Assay (BKA)

The bacterial killing assay, as the name indicates, assesses the potency of each peptide to kill the treated bacteria. Pathogens are incubated with 2-fold dilutions of each peptide for 1 hour then plated on suitable agar. CFUs are counted to determine the concentration remaining after peptide treatment. Figure 3 shows the dose-dependent bacterial reduction of *F. tularensis* SchuS4 in percentage after peptide treatment. Both WLBU2 and WR12 outperformed LL37 against SchuS4. WLBU2 attained 95% killing at a concentration of 3μM and 85% killing was achieved using 100 μM of WR12 (Table 2). The minimum bactericidal concentration (MBC), defined as the peptide concentration needed to kill 99% of the bacteria, was not achieved with any of the peptides. Statistical analysis revealed significant differences between WLBU2 and LL37 at concentrations

≥1.5μM and between WR12 and LL37 at concentrations ≥25μM ($P < 0.0001$). Moreover, WLBU2 had significantly better antimicrobial activity against SchuS4 than WR12 at concentrations ≤50μM ($P < 0.0001$). Thus, WLBU2 proved to be the best candidate between the tested peptides against SchuS4.

Next, I used the BKA to assess the antimicrobial activity of WLBU2 and WR12 against *B. pseudomallei*. Figure 4 shows the dose-dependent bacterial reduction in percentage after peptide treatment. Results show that WR12 and LL37 were unable to reduce bacterial counts of *B. pseudomallei*, while WLBU2 achieved a mean of 45% killing at 100μM (Figure 4A, Table3). Although statistical analysis indicates a significant increase in antimicrobial activity for WLBU2 compared to LL37, a 45% reduction in bacterial count is considered a modest activity.

In a study done by Kanthawong *et al* (2009) using potassium phosphate buffer (PPB) rather than PBS in the BKA, LL37 achieved 75% - 100% killing of 22 out of 24 different isolates of *B. pseudomallei* [70]. Therefore, I repeated the BKA of *B. pseudomallei* using PPB as my media. As Figure 4B indicates, LL37 and WR12 failed to reduce bacterial counts as before but WLBU2 activity increased to 60% killing at 25μM.



Bacterial cultures ($2x10^6$ c.f.u/ml) grown in BHI were treated with two-fold dilutions of the peptide in PBS. Percent reduction in bacterial count (y axis) upon treatment is plotted as a function of peptide concentration (x axis). Two-way analysis of variance (ANOVA) indicated a (*) significant difference at concentration $\geq$ 1.5μM, p <0.0001 between WLBU and LL37, a (**) significant difference at concentration $\geq$ 25μM, p < 0.0001between WR12 and LL37, and a (***) significant difference at concentration $\leq$ 50μM, p < 0.0001 between WLBU2 and WR12. The data shown is a result of two independent experimental trials.

Figure 3 Dose-dependent killing of F. tularensis by WLBU2, WR12 or LL37.

Table 2 Percentage killing of *F. tularensis* by WLBU2, WR12 or LL37.

Francisella tularensis			
%killing	**WLBU2 (μM)**	**WR12 (μM)**	**LL37 (μM)**
85%	< 1.5	100	> 100
90%	3	> 100	>100
95%	>3	> 100	> 100

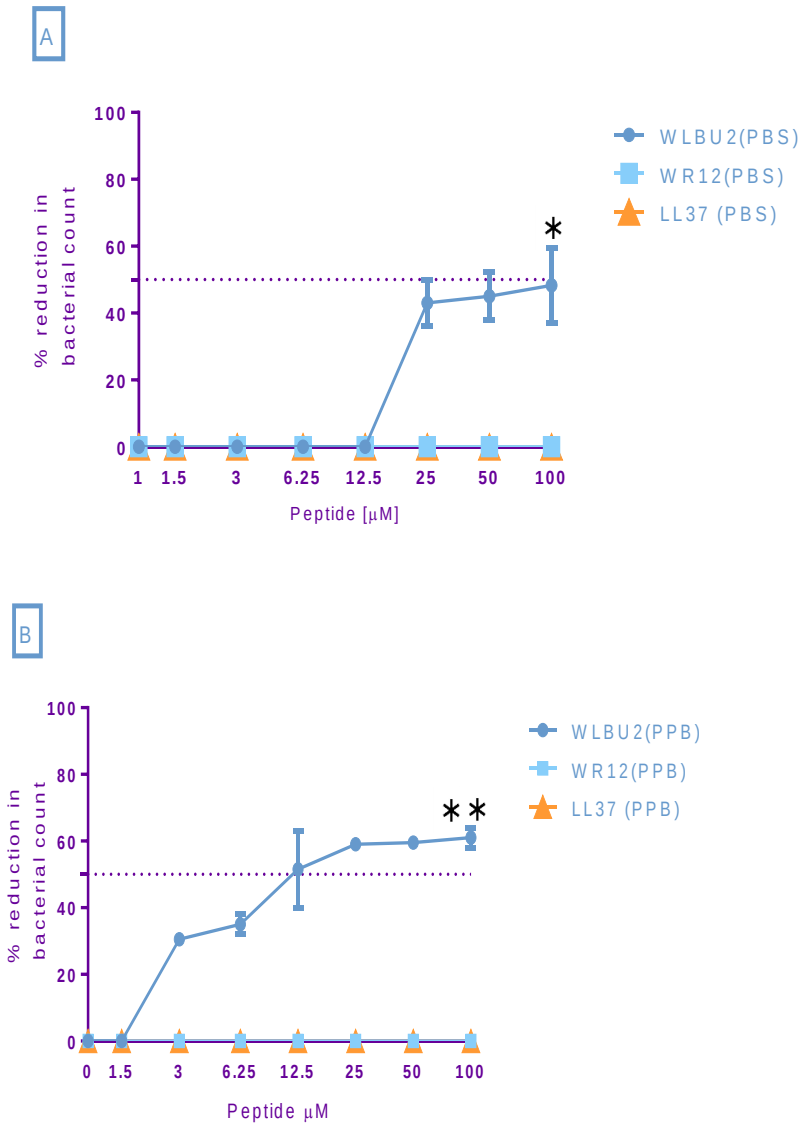
Peptide concentrations are summarized according to their ability to achieve 85, 95 and 99% killing of the bacteria.

Having demonstrated the activity of WLBU2 and WR12 against *F. tularensis* and *B. pseudomallei*, activity against *Y. pestis* was assessed. Figure 5 shows a dose-dependent reduction of *Y. pestis* after peptide treatment. Both WLBU2 & WR12 were significantly more effective at killing *Y. pestis* than LL37 with a 95% reduction in bacterial count at a concentration of 25μM of WR12 and 100μM of WLBU2 (Table 4). Statistical analysis revealed significant difference between WLBU2 and LL37 at concentrations ≥3μM and between WR12 and LL37 at concentrations ≥1.5μM ($P < 0.0001$). No significant difference between WLBU2 and WR12 was found against *Y. pestis* although, WR12 was able to achieve an MBC at a concentration of 50 μM. In agreement with the results shown here, a study done by O'Loughlin *et al* (2010) found that LL37 had minimal antimicrobial effect on *Y. pestis* due to bacterial modification of the outer membrane lipids decreasing its net negative charge [71].

For the following experiments with *F. tularensis* BHI medium was substituted with Chamberlain's Chemically Defined Medium (CCDM) due to unexpected difficulties with bacterial overnight growth in BHI. Previously, incubation of bacteria for 17 – 19 hours in BHI puts the bacterial growth in the exponential phase confirmed by an OD reading of 0.9 – 1 at 600mm. But since December 2013, *F. tularensis* stopped growing at the previous rate reaching a maximum OD

of 0.5 after 19 hours incubation. Several attempts to restore the previous growth seen in BHI were made but were unsuccessful. These included: BHI from different vendors, confirming pH using different pH meters and test papers, alternative sources of distilled water, adding cysteine, ferric pyrophosphate, or Isovitalex (supplements typically used in other media that supports Francisella growth). Growth in CCDM was not altered. Therefore, CCDM replaced the BHI as the media used for the overnight culture of *F. tularensis*.

Looking back at Figure 3, within the range of WLBU2 doses tested against *F. tularensis* it did not achieve the MBC. A likely explanation was saturation of the peptide required additional peptide to achieve the MBC. Figure 6 shows the comparison between the dose-dependent bacterial reduction in percentage after WLBU2 treatment with one and two doses. Rather than plating after 1 hour of incubating the bacteria with the peptide, a second dose of peptide at the same concentration tested was added and the plate was incubated for another hour. Clearly, multiple dosing of WLBU2 achieved significantly better antimicrobial activity with an MBC of 50 μM (Table 5).



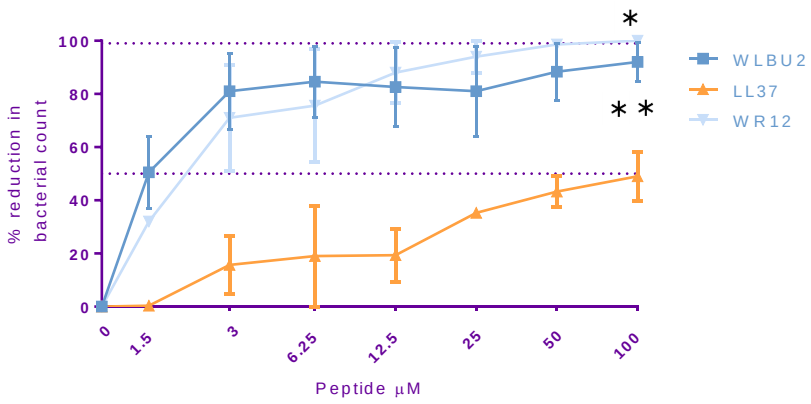
Bacterial culture ($1.2x10^6$ c.f.u/ml) grown in BHI were treated with two-fold dilutions of the peptide in A. PBS or B. PPB. Percent reduction in bacterial count (y axis) upon treatment is plotted as a function of peptide concentration (x axis). Two-way analysis of variance (ANOVA) of WLBU2 (PBS) versus LL37 (*) and WLBU2 (PPB) versus LL37 (**) was performed. (*) significant difference at concentration 25, 50 and 100μM, $p < 0.0001$. (**) significant difference at concentration ≥ 3μM, $p < 0.0001$. The data shown is a result of three independent experimental trials.

Figure 4 Dose-dependent killing of B. pseudomallei by WLBU2, WR12 or LL37.

Table 3 Percentage killing of *B. pseudomallei* by WLBU2, WR12 or LL37.

B. pseudomallei			
%killing	**WLBU2 (μM)**	**WR12 (μM)**	**LL37 (μM)**
85%	>100	>100	>100
90%	>100	>100	>100
95%	>100	>100	>100

Peptide concentrations are summarized according to their ability to achieve 85, 95 and 99% killing of the bacteria



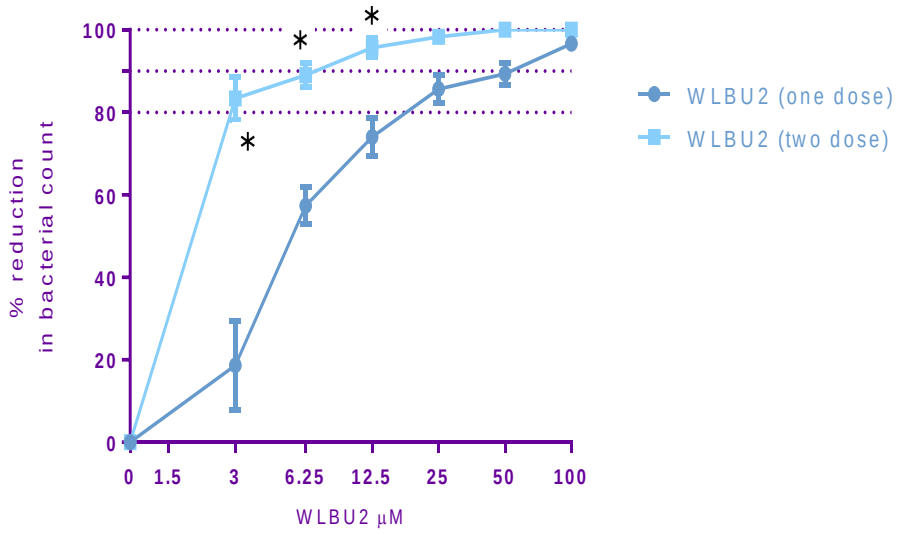
Bacterial cultures ($2x10^6$ c.f.u/ml) grown in BHI were treated with two-fold dilutions of the peptide in PBS. Percent reduction in bacterial count (y-axis) upon treatment is plotted as a function of peptide concentration (x axis). Two-way analysis of variance (ANOVA) of WR12 versus LL37 (*) and WLBU2 versus LL37 (**) was performed. (*) significant difference at concentration ≥ 3μM, $P < 0.0001$. (**) significant difference at concentration ≥ 1.5μM, $P < 0.0001$. The data shown is a result of three independent experimental trials.

Figure 5 Dose-dependent killing of Y. pestis by WLBU2, WR12 or LL37.

Table 4 Percentage killing of *Y. pestis* by WLBU2, WR12 or LL37.

Y. pestis			
%killing	**WLBU2 (µM)**	**WR12 (µM)**	**LL37 (µM)**
85%	6.25	< 12.5	> 100
90%	<100	< 25	>100
95%	100	25	> 100

Peptide concentrations are summarized according to their ability to achieve 85, 95 and 99% killing of the bacteria



Bacterial cultures ($1x10^6$ c.f.u/ml) grown in CCDM were treated with two-fold dilutions of the peptide in PBS. Percent reduction in bacterial count (y-axis) upon treatment is plotted as a function of peptide concentration (x axis). Two-way analysis of variance (ANOVA) of WLBU2 (one dose) versus WLBU2 (two doses) was performed. (*) significant difference at concentrations 3, 6.25 and 12.5µM, $P < 0.0001$. The data shown is a result of three independent experimental trials.

Figure 6 Dose-dependent killing of F. tularensis by one dose and two doses of WLBU2.

Table 5 Percentage killing of *F. tularensis* by one or two consecutive doses of WLBU2.

Francisella tularensis		
%killing	**WLBU2 (μM) One Doses**	**WLBU2 (μM) Two Doses**
85%	25	< 3
90%	50	6
100%	> 100	50

Peptide concentrations are summarized according to their ability to achieve 85, 90 and 99% killing of the bacteria

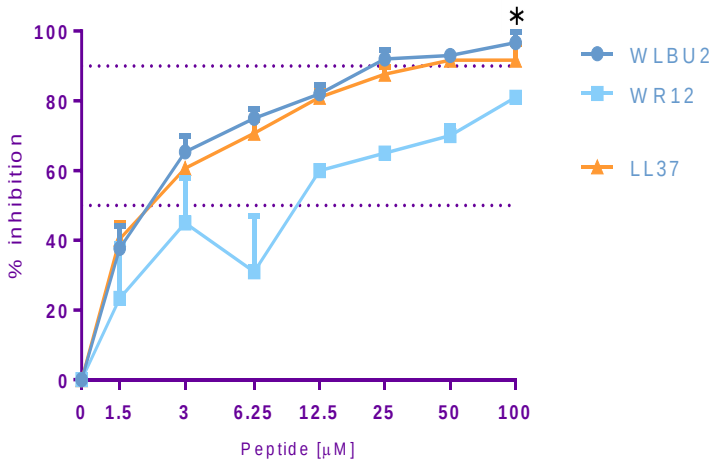
4.2.2 Growth inhibition assay (GIA)

The growth inhibition assay is an *in vitro* test that depends on the measurement of turbidity as an indicator of microorganism population. Deslouches *et al* demonstrated the usefulness of this assay in studying the antimicrobial effects of several peptides on *P. aeruginosa* and MRSA [66]. Using this assay, bacterial growth is monitored after addition of the peptides in growth media with the bacteria for 24hr giving us an indication as to their antimicrobial activity. Therefore, in the effort to properly evaluate the antimicrobial effect of WLBU2 and WR12 on all three pathogens, a growth inhibition assay was performed.

Figure 7 shows the dose dependent inhibition of *F. tularensis* growth by WLBU2, WR12 and LL37. As predicted, WLBU2 had the highest growth inhibition with a minimum inhibitory concentration (MIC) of 25 μM (Table 6). WR12 had moderate activity at concentrations ≥12.5μM but failed to reach MIC at the highest dose tested (100 μM). WLBU2 had significantly higher activity than WR12 between 3 μM and 50μM ($P<0.0001$). On the other hand, LL37 performed better compared to the results obtained in the BKA with a MIC of 50 μM. The difference between

the BKA and GIA might be a result of the difference in the liquid media (PBS for the BKA; BHI for the GIA) between the two *in vitro* assays. As mentioned before, in a GIA bacteria are incubated with the peptides in a growth medium rather than PBS. Certain components in the BHI might enhance the activity of LL37 against *F. tularensis.* Another explanation could be that the bacteria in this assay are incubated with the peptides for 24hr compared to 1 hour in a BKA. Although studies have shown that antimicrobial peptides rapidly kill *P. aeruginosa* within 30 – 180 seconds [65], a kinetic analysis of WLBU2, WR12 and LL37 specifically against *F. tularensis* is needed. Further, in the BKA assay the peptide is interacting with static bacteria in comparison to the metabolically active bacteria in culture media in the GIA assay. The difference in bacterial membrane dynamics and its stability between the two assays could also justify the variable LL37 results.

Figure 8 shows the dose – dependent inhibition of *B. pseudomallei* growth by WLBU2, WR12 or LL37. Similar to the BKA results, none of the peptides were able to achieve their MIC within the concentrations tested (Table 7). LL37 activity is enhanced, as seen before with *F. tularensis*, and a moderate increase in WR12 activity is seen as well. Statistical analysis indicates no difference in activity between the three peptides. All were able to reach approximately a 50% reduction in growth at the highest concentration tested.



Bacterial cultures (2X10^6 c.f.u/ml) grown and treated in BHI with twofold dilutions of the peptide. Percent inhibition in bacterial growth (y-axis) upon treatment is plotted as a function of peptide concentration (x axis). Two-way analysis of variance (ANOVA) of WR12 versus WLBU2 (*), WLBU2 versus LL37 and WR12 versus LL37 (**) was performed. (*)(**) significant difference at concentrations between 3 µM and 50µM, $P < 0.0001$. The data shown was a result of three independent experimental trials. Each trial was done in triplicates.

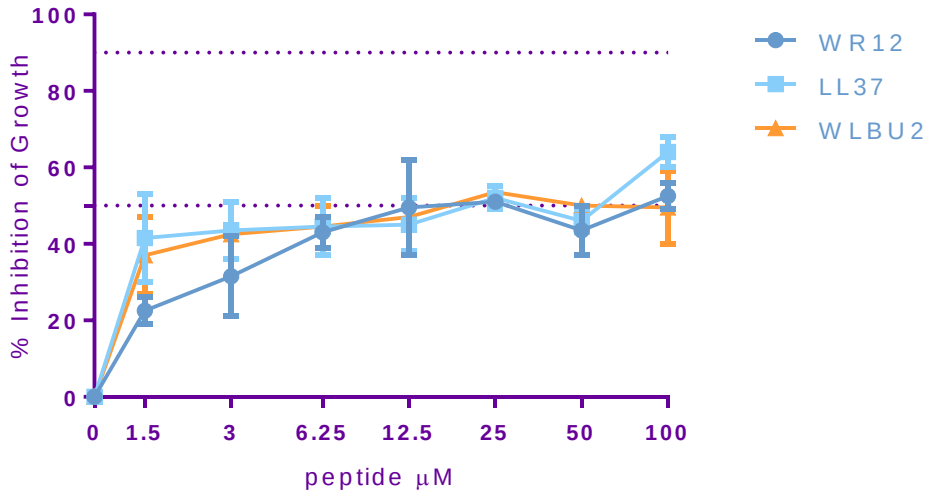
Figure 7 Dose – dependent inhibition of F. tularensis growth by WLBU2, WR12 or LL37.

Table 6 Percentage inhibition of *F. tularensis* by WLBU2, WR12 or LL37.

Francisella tularensis			
%Inhibition	**WLBU2 (µM)**	**WR12 (µM)**	**LL37 (µM)**
85%	<25	>100	25
90%	25	>100	50
95%	<100	>100	>100

Peptide concentrations are summarized according to their ability to achieve 85, 95 and 99% inhibition of the bacterial growth.

The WLBU series of peptides is composed of peptides with the same composition but in different lengths. Deslouches *et al* studied the optimal length and concluded that WLBU2 with its 24 residues was the lead compound against *P. aeruginosa* and MRSA [63]. Due to the modest activity of WLBU2 against *B. Pseudomallei*, the longer peptides WLBU3 & WLBU4 were tested using the growth inhibition assay.



Bacterial cultures ($1.2X10^6$ c.f.u/ml) grown and treated in BHI with two-fold dilutions of the peptide. Percent inhibition in bacterial growth (y-axis) upon treatment is plotted as a function of peptide concentration (x axis). Two-way analysis of variance (ANOVA) of WR12 versus WLBU2, WLBU2 versus LL37 and WR12 versus LL37 was performed. No statistical difference was found. The data shown was a result of three independent experimental trials. Each trial was done in triplicates.

Figure 8 Dose – dependent inhibition of B. pseudomallei growth by WLBU2, WR12 or LL37.

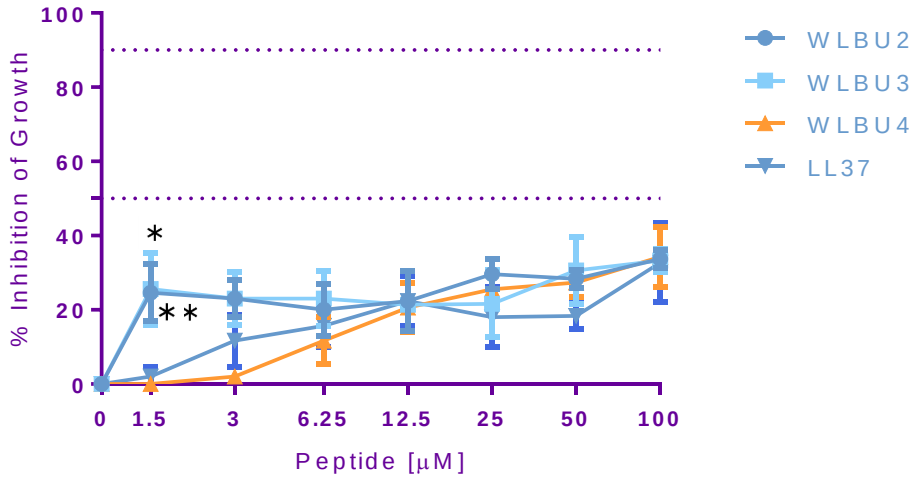
Table 7 Percentage inhibition of *B. pseudomallei* by WLBU2, WR12 or LL37.

B. pseudomallei			
%Inhibition	**WLBU2 (µM)**	**WR12 (µM)**	**LL37 (µM)**
85%	>100	>100	>100
90%	>100	>100	>100
95%	>100	>100	>100

Peptide concentrations are summarized according to their ability to achieve 85, 95 and 99% inhibition of the bacterial growth.

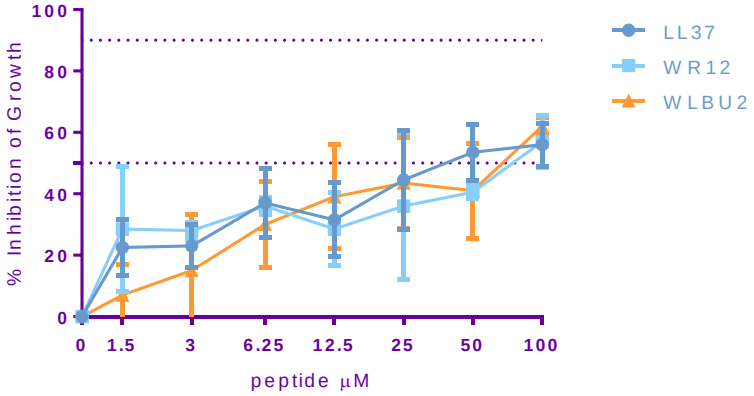
Figure 9 shows the dose dependent growth inhibition in percentage after treatment with each peptide. There was a slight difference between the growth inhibition of the three WLBU peptides with WLBU2 and WLBU3 having a significant increase of activity compared to either LL37 or WLBU4 at concentration 1.5µM. No significant difference was found between WLBU2 and WLBU3, thus increasing the length of the peptides did not improve activity against *B. pseudomallei*.

As for *Y. pestis*, Figure 10 shows the dose-dependent inhibition of growth by WLBU2, WR12 or LL37. Contrary to the results obtained from the BKA, none of the peptides were able to achieve MIC, and there were no significant differences between them (Table 8). Again that might be due to the effect of the media used for overnight growth on the peptides' antimicrobial activity or bacterial surface composition.



Bacterial cultures ($1.2X10^6$ c.f.u/ml) grown and treated in BHI with twofold dilutions of the peptide. Percent inhibition in growth (y-axis) upon treatment is plotted as a function of peptide concentration (x axis). Two-way analysis of variance (ANOVA) between WLBU2, WLBU3, WLBU4 and LL37 was performed. (*) Indicates Statistical difference between WLBU2 and WLBU4, WLBU2 and LL37 at 1.5µM. (**) indicates statistical difference between WLBU3 and LL37, WLBU3 and WLBU4 at 1.5µM. The data shown was a result of three independent experimental trials. Each trial was done in triplicates.

Figure 9 Dose – dependent inhibition of B. pseudomallei growth by WLBU2, WLBU3, WLBU4 or LL37.



Bacterial cultures ($2X10^6$ c.f.u/ml) grown and treated in Bacto nutrient media with twofold dilutions of the peptide. Percent inhibition in bacterial growth (y-axis) upon treatment is plotted as a function of peptide concentration (x axis). Two-way analysis of variance (ANOVA) of WR12 versus WLBU2, WLBU2 versus LL37 and WR12 versus LL37 was performed. No statistical difference was found. The data shown was a result of two independent experimental trials. Each trial was done in triplicates.

Figure 10 Dose – dependent inhibition of Y. pestis growth by WLBU2, WR12 or LL37.

Table 8 Percentage inhibition of *Y. pestis* by WLBU2, WR12 or LL37.

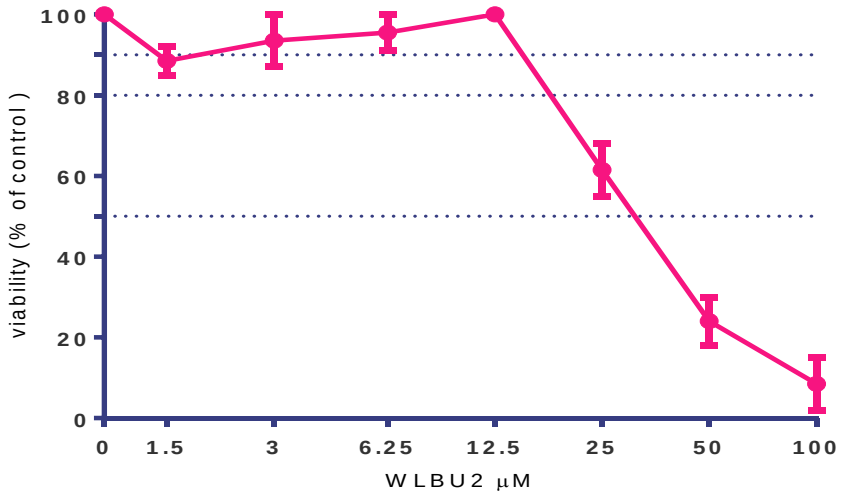
Y. pestis			
%Inhibition	**WLBU2 (µM)**	**WR12 (µM)**	**LL37 (µM)**
85%	>100	>100	>100
90%	>100	>100	>100
95%	>100	>100	>100

Peptide concentrations are summarized according to their ability to achieve 85, 95 and 99% inhibition of the bacterial growth.

4.3 Antimicrobial activity of WLBU2 against *F. tularensis* infected macrophages

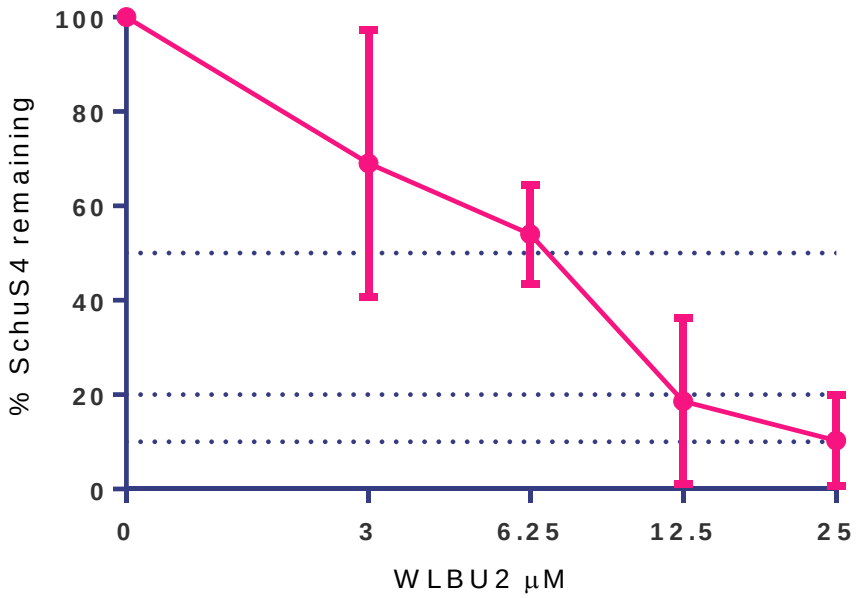
Many CAPs act directly by permeabilizing the membrane of the microbial targets without interacting with any specific receptors. Thus, their effect on mammalian cells is a concern considering that some peptides have shown potentially harmful side effects such as lysis of red blood cells [72]. For the *ex vivo* experiments with eCAPs, a transformed mouse macrophage cell line (J774) was infected with *F. tularensis*. Transformed cells are more sensitive to membrane-permeabilizing peptides due to higher phospatidylserine content[73]. Before moving on with the *ex vivo* experiments the cytotoxicity of WLBU2 on J774 macrophages was determined using a MTS test. As Figure 11 demonstrates WLBU2 treatment resulted in a dose-dependent decrease in cell viability with 50% of the cells lost at a concentration of 25μM. Therefore, only WLBU2 concentrations of 25μM or less were used in any *ex vivo* experiments.

F. tularensis is a facultative intracellular organism that invades and multiplies within macrophages [74, 75]. Thus, for a treatment to be successful it will have to act on the intracellular pathogen. To determine the intracellular antimicrobial effect of WLBU2, *ex vivo* bacterial clearance of SchuS4 from infected macrophage cells (J774) was assessed using concentrations ranging from 3μM to 25μM. J774 cells were infected with SchuS4 at an MOI of 1:100, washed, and then treated with different concentrations of WLBU2 (3μM to 25μM) for one hour. Cell lysate was plated on CHA plates to determine bacterial killing and bacterial colonies were counted after 48 hours of incubation. Figure 12 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 treatment with 12.5μM for an hour. These findings combined with the MTS test results suggest that at a dose of 12.5 μM the peptide is able to kill most of the intracellular bacteria with minimal harm to the mammalian cells.



Cell viability was determined using a MTS assay. Treatment of cells with WLBU2 resulted in a dose-dependent decrease in cell viability with 50% of the cells lost at a concentration of 25μM. Results of cell growth was measured relative to a control where J774 cells were grown in 100% media. The data shown was a result of three independent experimental trials.

Figure 11 J774 macrophage cells treated for 1 hour with a range of concentrations of WLBU2.



F. tularensis infected J774 cells (MOI 1:100) were treated with twofold dilutions of WLBU2 for 1 hour. Percent remaining of the bacteria (y-axis) upon treatment is plotted as a function of peptide concentration (x axis). The data shown was a result of three independent experimental trials.

Figure 12 Dose-dependent killing of intracellular F. tularensis by WLBU2.

5.0 DISCUSSION

The discovery of numerous natural cationic peptides in the last three decades and their antimicrobial role in our bodies has sparked the interest of many scientists. The growing understanding of the structure and function of such peptides led to the development of several eCAPs such as WLBU and WR series. Here the antimicrobial activity of WLBU and WR12 peptides was tested against three tier 1 select agents: *Francisella tularensis, Burkholderia pseudomallei* and *Yersinia pestis.* These bacterial agents are considered tier 1 agents due to their potential for misuse, high fatality rate if not treated appropriately, and the development of antibiotic resistance to their current effective treatment.

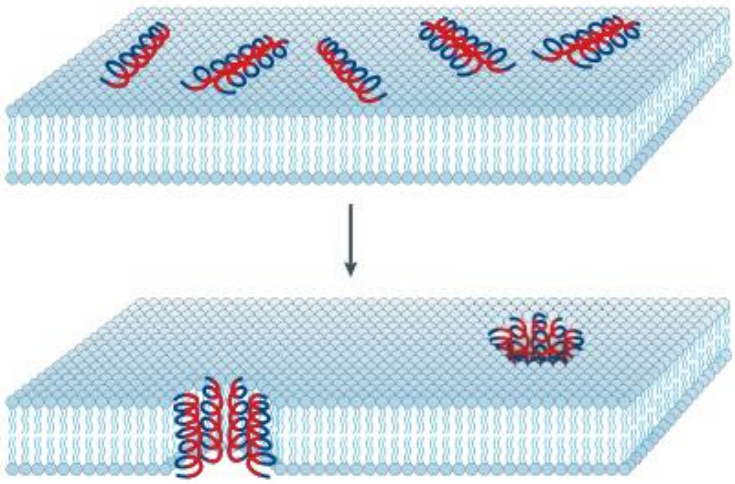
Previous studies documented the superior antimicrobial activity of WLBU2 and WR12 against several bacterial agents such as *P. aeruginosa* and MRSA [65-68]. In this study, *in vitro* tests of WLBU2 and WR12 also demonstrated superior antimicrobial effect against *F. tularensis* and *Y. pestis* when compared to the natural peptide (LL37). WLBU2 proved to be the best candidate against *F. tularensis* with a single dose of 3μM needed to achieve a 90% reduction in bacterial count and a MIC of 25 μM. Both WLBU2 and WR12 achieved similar results against *Y. pestis*, but only WR12 was able to attain a MBC of 50 μM.

B. pseudomallei provided a greater challenge with only moderate susceptibility to WLBU2. Nevertheless, WLBU2 antimicrobial effectiveness against *B. pseudomallei* surpasses that of LL37. It was also observed that results depended on the testing environment. A substantial reduction in bacterial count was seen with WLBU2 in potassium phosphate buffer but not in phosphate buffered saline. The role of PPB in enhancing the peptides' antimicrobial function is still unclear. PPB may

affect the bacterial membrane enhancing its disruption by the peptide or create a more suitable environment for peptide activity. The difference in outcome between my experiments and the Kanthawong *et al* study concerning LL37 might be related to the *B. pseudomallei* isolates used. The isolate used in this study, 1026b, is considered a "prototypical" lab strain isolated from a diabetic 29-year-old female patient with septicemic melioidosis. While in Kanthawong *et al*, 13 isolates were from patients admitted to Srinagarind Hospital (Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand), and the other 11 were from soil collected from the northeastern endemic region of Thailand [70]. It is well established that there are different LPS structures of *B. pseudomallei* depending on the isolate: smooth type A, smooth type B, rough type A or rough type B LPS structures [76]. Since LPS form a major part of the outer membrane of the bacteria and shields it from antimicrobial attacks, such variation might contribute to the variation in bacterial susceptibility to peptides [77]. Additional complexity to bacterial surfaces is generated by production of surface polysaccharide capsules. Bacterial capsule is known to hinder complement activation and phagocytosis of *B. pseudomallei* [78]. Wikraiphat *et al* further showed that capsule mutants of this bacterium survived poorly in macrophages and were more susceptible to antimicrobial killing by histatin and lactoferrin when compared to the wild type [79]. Also, Hayden et al suggested that genetic changes that occur in subpopulations of *B. pseudomallei* during acute infections and post-treatment are not found in 1026b which could contribute to the variance seen in the antimicrobial activity of the peptides [80].

The mechanism of action of these peptides is still under investigation but the general assumption is that their antimicrobial action stems from their ability to attach and disrupt the microbial outer membrane leading to depolarization, metabolite leakage, and ultimately death of the pathogen (Figure 13). 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 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, Galvan *et al* demonstrated that the susceptibility of *Y. pestis* to CAPs such as cathelicidin is mediated by the capsular antigen fraction 1 [81]. Also, Vonkavaara *et al* illustrated the importance of surface lipid A and Kdo core for interactions of *F. tularensis* with CAPs. On the other hand, Madhongsa *et al* demonstrated that the MIC value of several peptides such as polymyxin B for *B. pseudomallei* is several magnitudes higher than those of *P. aeruginosa* or *E. coli* due to the type of LPS moiety on its outer membrane [76]. Further, *B. pseudomallei* resists treatment by forming micro-colonies using its glycol-calyx polysaccharide capsule and creating a protective environment against the peptide. This might explain eCAPs enhanced activity against *F. tularensis* and *Y. pestis* in comparison to the moderate antimicrobial effect of WLBU2 and failure of WR12 against *B. pseudomallei*.



In this model the attached peptides aggregate and inser into the bacterial membrane so that hydrophobic peptide regions align with the lipid core region and the hydrophilic peptide region form the interior region of the pore. Hydrophilic regions of the peptide are shown coloured red, hydrophobic regions of the peptide are shown coloure blue. Reprinted by permission from Macmillan Publishers Ltd: Nature [82], copyright 2005.

Figure 13 A model of antimicrobial peptides mechanism of action.

It important to note that the antimicrobial activity of WLBU2 was affected by the media used in overnight cultures of bacteria. *F. tularensis* grown in BHI was more susceptible to WLBU2 with 90% reduction at 3μM compared to 50 μM for the bacteria grown in CCDM. Hazlett et al have shown that SDS-PAGE analysis of *F. tularensis* grown in CCDM or Muller Hinton Broth (MHB) displayed different protein profiles from those grown in BHI. While *F. tularensis* grown in BHI had similar mRNA profile to the bacteria isolated from macrophages, MHB and CCDM yield bacteria that are phenotypically different with abundant outer membrane proteins that are usually down-regulated during host adaptation [83]. This difference might be due to the high level of free amino acids in MHB (120 mM) and CCDM (75mM) affecting the protein production in bacteria. Further, Cherwonogrodzkey et al demonstrated that when encapsulated LVS was passaged in CCDM it created a thicker capsule and increases in virulence [84]. Because CAPs interact with bacterial surface, variation in the outer membrane proteins and capsule production by different media might be responsible for the difference in WLBU2 activity against *F. tularensis*.

In practice, antibiotics are usually given in multiple doses to maintain a drug concentration capable of ridding the infected body from the pathogens. When a single dose of WLBU2 was tested against *F. tularensis*, it was unable to achieve a MBC and leveled off at 97% reduction in bacterial count. We theorized that saturation of WLBU2 was accounting for the inability of WLBU2 to achieve the MBC (defined as ≥99% killing). With that in mind, WLBU2 was tested *in vitro* with 2 consecutive doses at the same concentration. Compared to a single dose, WLBU2 multiple dosing proved to be more effective with complete killing of *F. tularensis* at 50 μM (MBC=50 μM). Basically, the initial dose of 50 μM WLBU2 eliminated more than 90% of the bacteria making it easier for the second dose to achieve complete killing. Also, WLBU2 multiple dosing performed better than the single dosing over the whole gradient of doses. This further

confirms the assumption that saturation of the peptide had occurred, requiring additional peptide to achieve higher killing activity and that bacteria surviving the first dose of peptide were not resistant to WLBU2.

During the generation of the WLBU series, peptides were tested against *P. aeruginosa* to determine the length for optimal antimicrobial activity. It was thought that longer peptides would have higher activity due to their greater membrane-spanning capacity. Nevertheless, WLBU2 with its 24 residues had the ideal length against *P. aeruginosa* with no noticeable increase in activity with WLBU3 (36-residue) and WLBU4 (48-residue) [63]. Similarly, when testing WLBU2, 3 and 4 against *B. pseudomallei* there were no noticeable difference in the growth inhibition of the three peptides against this pathogen. Thus, *B. pseudomallei* is able to resist the antimicrobial effect of the WLBU series regardless of their length. Madhongsa *et al* emphasized the structure activity relationship of the antimicrobial peptides and its importance against *B. pseudomallei*. Using an isolate with high biofilm production (H777), Madhongsa *et al* demonstrated the higher efficacy of cyclic bacitracin against *B. pseudomallei* compared to other linear αhelix peptides [76]. Therefore, greater membrane permeabilization ability against our tested isolate might be achieved not by increasing the length of the our peptides but by higher order of oligomers and cyclic structures

In light of the evident antibacterial efficacy of WLBU2 *in vitro*, it is vital to know whether this peptide displays any toxicity to mammalian cells or not. Deslouches et al demonstrated that concentrations up to 30μM of WLBU2 can damage the bacterial cells while sparing leukocytes and human skin fibroblasts [64]. This antimicrobial selectivity may be attributed to the difference in lipid content between eukaryotic and bacterial cell membranes. Due to the presence of cholesterol in eukaryotic membranes they tend to form weaker hydrophobic interactions with

eCAPs, while the negatively charged bacterial membranes forms a strong electrostatic interaction with the them [85].

F. tularensis is an intracellular organism that invades and replicates in macrophages. Accordingly, for the peptides to be effective *in vivo* they would have to exert their antimicrobial activity on intracellular pathogens with minimal effect on the macrophages. From the MTS staining assays results it was concluded that while WLBU2 could be toxic for J774 cells, under the concentration of 25μM WLBU2 had minimal toxic effect on J774 macrophages. Taking that into consideration, all *ex vivo* infected macrophages were treated with concentrations of less than 25μM of WLBU2. WLBU2 demonstrated a high antimicrobial efficacy against intracellular bacteria with only 20% bacteria remaining after treatment with 12.5μM for an hour. Despite our limited knowledge about the pharmacokinetics and dynamics of these peptides, results of the *ex vivo* experiments using WLBU2 against *F. tularensis* infected macrophages suggest that the peptide is able to kill the intracullar bacteria with minimal harm to the mammalian cells.

Several factors should be considered when these peptides are tested in *in vivo* settings, including the concentrations of antimicrobial peptides at the sites of infection, inhibiting or synergistic substances present in tissues and fluids (i.e. lysozyme, natural antimicrobial peptides and physiological salt concentrations). Deslouches et al demonstrated that the WLBU2 had prophylactic and therapeutic effects against *P. aeruginosa* bacteraemia in mice; 3 and 4 mg/kg given 1 hour post-infection was able to increase survival rates compared to control [65]. Therefore, for future directions I would suggest assessing the antimicrobial activity and toxicity of WLBU2 in a mouse model for tularemia. Balb/c mice can be infected via the respiratory tract by aerosol delivery of SchuS4 and then treated with i.v. (intravenous) or i.n. (intranasal) WLBU2. Mice weight and temperature should be monitored daily to evaluate the effectiveness of WLBU2 in

clearing the infection. Also the same model can be used to assess the benefits of prophylactic and multiple dosing of the peptide.

In conclusion, there are three major requirements for any successful antimicrobial peptides: potency, specificity and activity under physiological conditions. In this study we demonstrated the potency of WLBU2 and its selectivity for *F. tularensis*. Further investigation is warranted, but WLBU2 demonstrates a great potential as a broad spectrum antimicrobial agent against *F. tularensis*. As for *B. pseudomallei* and *Y. pestis*, results were inconclusive about the usefulness of WLBU and WR12 against these pathogens. Nevertheless, eCAPs could be useful as a broad-spectrum countermeasure in situations concerning public health, such as bioterror attacks, until the causative agent is identified.

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