DE NOVO CATIONIC ANTIMICROBIAL PEPTIDES AS THERAPEUTICS AGAINST *PSEUDOMONAS AERUGINOSA*

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Cationic antimicrobial peptides (CAPs) are a very diverse group of amphipathic agents that demonstrate broad activity against Gram-positive and -negative bacteria. To overcome the obstacle of drug resistance among bacterial pathogens, CAPs have been extensively investigated as a potential source of new antimicrobials with novel mechanisms of action that may complement current antibiotic regimens. However, the suppression of antimicrobial activity in biological conditions (*e.g.*, physiological salt concentrations, serum) constitutes a major challenge to the successful development of CAPs for clinical applications. *We hypothesized that CAPs with optimized amphipathic structures can be designed* de novo *to enhance antibacterial activity and selective toxicity in environments that are generally challenging to host-derived peptides*. F*urthermore, the antibacterial efficacy will positively correlate with length, charge, Trp content, and helicity.*

Three specific aims were developed to address this hypothesis. The first aim addressed the design of amphipathic peptides to evaluate the influence of helicity, length, and Trp content on activity. Using a base unit peptide approach, we synthesized a multimeric series of 12-residue lytic base unit (LBU) composed of Arg and Val residues, positioned to form idealized amphipathic α-helices. Another series of LBU derivatives (WLBU) was engineered by

substituting Trp residues on the hydrophobic face. The correlation between length and helicity was established by circular dichroism analysis. Bacterial killing assays revealed no appreciable increase in activity for peptides longer than 24 residues. In addition, the inclusion of Trp residues in the hydrophobic face increased potency and selectivity in a novel co-culture system utilizing bacteria and primary cell lines, which led to the selection of WLBU2 as the shortest peptide (24 residues in length) with the highest potency. The second objective was to examine the selective toxicity of WLBU2 in biological or biologically-derived media. In contrast to the human CAP LL37, the peptide WLBU2 displayed potent activity against *Pseudomonas aeruginosa* in the presence of human serum and human blood *ex-vivo*, with no detectable red blood cell lysis or toxicity to human monocytes at all test concentrations. Finally, we demonstrated potent activity of WLBU2 in intraperitoneal and intravenous mouse models of *Pseudomonas aeruginosa* infection. WLBU2 not only protected mice prophylactically but also eradicated *P. aeruginosa* from the blood and other tissues at 3 to 4mg/kg. Furthermore, WLBU2 displayed only a minor stimulatory effect on inflammatory cytokines, notably IL1-β and TNF-α. Consistent with our *in vitro* studies, the *in vivo* data provide strong evidence for the potential application of WLBU2 in the treatment of systemic infection due to *P. aeruginosa*.

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1. CHAPTER 1: Introduction

1.1. The impact of infectious diseases

Infectious diseases constitute a worldwide health crisis. Until the 20th century, the treatments of many life-threatening microbial diseases (*e.g.*, tuberculosis, the bubonic plague) had been highly inadequate (6, 21, 36, 56, 64, 150, 165, 174, 213, 236, 332, 344). However, the development of safe and effective vaccines and antibiotics, coupled with amelioration of sanitation, resulted in a dramatic decline in infection-related fatalities from 1900 to 1980 (22, 26, 29, 33-35, 40, 61, 102, 110, 182, 230). This enormous improvement in the control of infectious diseases led to the belief that humans were winning the centuries-long war against infectious microbes. The resurgence of infection-related fatalities over the past 25 years, however, has shown that this optimism was premature (34, 35, 104, 132, 166, 215, 320, 380). Infectious diseases remain the world's leading cause of death, ranking third among causes of mortality in the United States (134, 389).

Antibacterial drug resistance is one of the most critical issues in the management of infectious diseases (291). Bacteria are among the fastest growing organisms, which facilitates rapid genetic changes and the selection of traits allowing adaptation to constantly changing ecosystems. The emergence of new phenotypes occurs by alteration of native genes or by transfer of genes among organisms (23, 71, 288). These processes are often associated with the acquisition of new virulence factors and resistance to currently used antibiotics. Hence, organisms re-invent themselves, leading to re-emerging pathogens that may require the development of novel therapeutics for eradication (258).

Multiple drug resistant (MDR) bacteria have emerged in response to selective pressure created by the widespread use of antibiotics (83). The propagation of such organisms may occur in hospitals (nosocomial infections), specialized health centers (*e. g.*, cystic fibrosis centers), and communities where antibiotics are used inappropriately (77, 130, 348). Between 1980 and 1992, respiratory tract infections accounted for the largest portion of infection-related mortality (28/100,000) in the United States, with an increased death rate from 20 to 30 per 100,000 (35, 66). Although acquired immune deficiency syndrome or AIDS-related illnesses are among the major, but indirect, causes of this increase in fatalities, antibiotic resistance remains a primary concern (137, 291).

1.2. *Pseudomonas aeruginosa* and antibiotic resistance

Among major MDR pathogens, a bacterial organism requiring special attention is *Pseudomonas aeruginosa*. This is an opportunistic Gram-negative aerobic rod that can be isolated in water, soil, plants, and animals. Because of its ubiquity, the potential for frequent exposure to hospitalized patients, and high tendency to become multiply resistant to antibiotics, *P. aeruginosa* poses a serious burden to the clinical management of bacterial diseases (85, 137). *P. aeruginosa* has a high propensity to develop a wide range of mechanisms of resistance, which include restricted uptake, drug efflux, and enzymatic modification.

1.2.1. Restricted Permeability

In comparison to other typical Gram-negative bacteria (*e.g.*, *E. coli*), *P. aeruginosa* is thought to be inherently less susceptible to several antibiotics by restricting their uptake (435). Clinical isolates of *P. aeruginosa*, namely cystic fibrosis (CF) patient isolates, have a high propensity to form an outermost polysaccharide or mucin layer called alginate, which gives *Pseudomonas* a smooth and mucoid appearance (Figure 1) (111, 232). The anionic property of this exopolysaccharide matrix may render this organism less sensitive to cationic antibiotics, including aminoglycosides and amphipathic peptides (*e.g.*, the polymyxins) (20). Furthermore, the outer membrane of *P. aeruginosa* is spanned by barrel-shaped proteins called porins, which are involved in the uptake of small hydrophilic molecules (amino acids, antibiotics, etc.) (220). The loss of function of some porin molecules ultimately leads to decreased permeability to specific antibiotics (*e.g.*, resistance to imipenem due to loss of oprD) (45, 136, 208, 220). Finally, *P. aeruginosa* has the property of forming biofilms or communities of bacteria that adhere to a solid phase. Bacteria existing in a biofilm tend to be less susceptible to antibiotics, leading to difficulty in eradicating the infections. Inside the host, biofilms may form on mucosal or epithelial surfaces, catheters, and other medical devices. Biofilm formation often leads to serious health problems, notably in individuals with chronic infections (*e.g.*, CF patients) and patients subjected to certain procedures that require the implantation of prosthetic medical devices (*e.g.*, central lines, urinary catheters, etc.) (43, 96, 108, 138, 168, 235, 292).

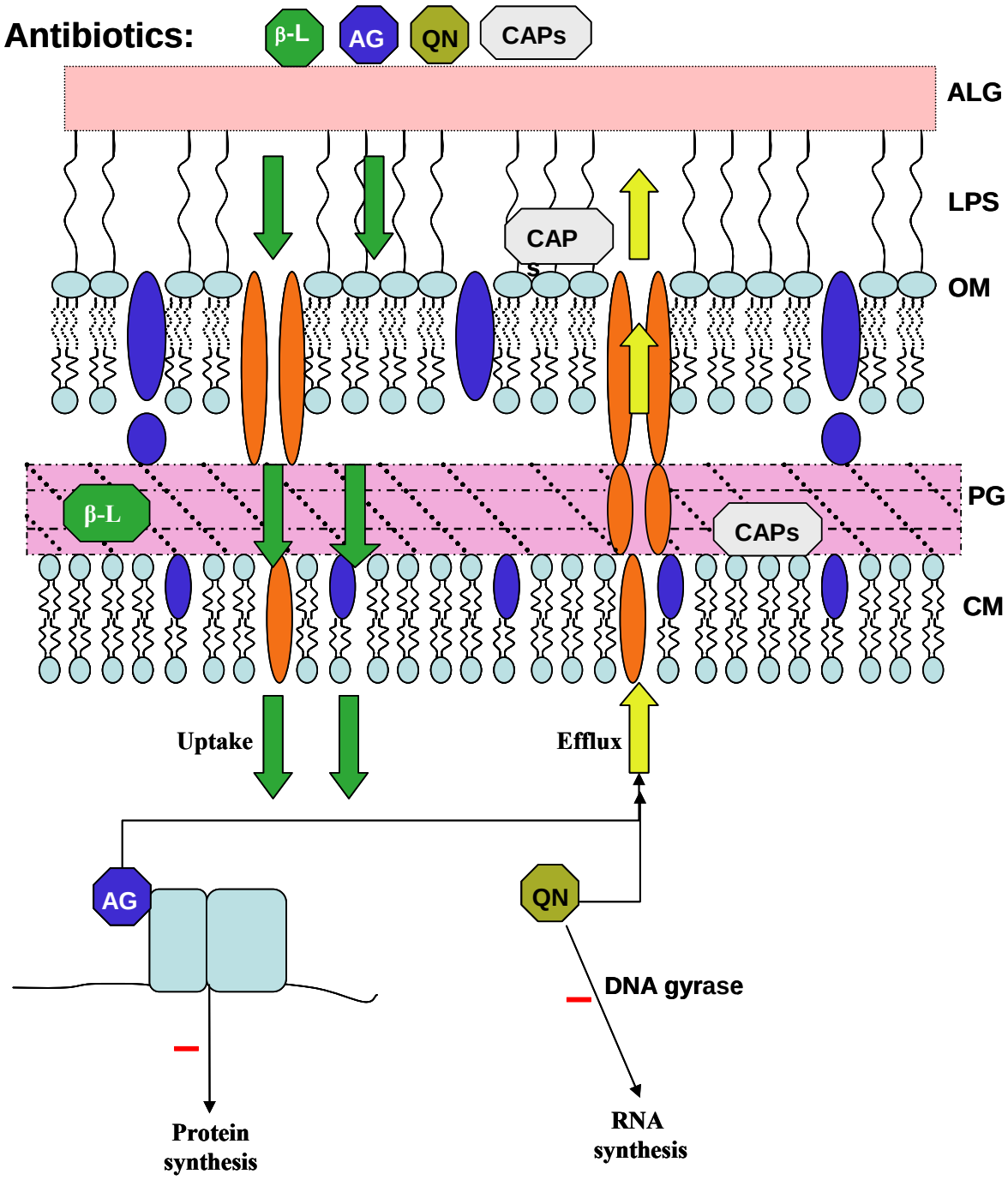


Figure 1. Surface of *P. aeruginosa* in relationship to antibiotic resistance (Adapted from Lambert, 2002)

Many clinical strains of *P. aeruginosa* may synthesize an outermost polysaccharide layer called alginate (ALG), which may selectively reduce drug permeability. To penetrate the cell, antimicrobial agents such must traverse this layer, the lipopolysaccharide (LPS)-containing outer membrane (OM), the proteoglycan layer (PG) where ß-lactam antibiotics are inactivated by ß-lactamases), and the cytoplasmic membrane (CM). Once inside the cells, antimicrobial agents

such as aminoglycosides (AG) and quinolones (QN) may be pumped out of the cells. CAPs: cationic antimicrobial peptides; ß-L: ß-lactam antibiotics.

1.2.2. Drug Efflux

In addition to the restricted permeability of the bacterial envelope, other effective mechanisms of resistance can be employed by *P. aeruginosa*. One way *P. aeruginosa* may display multiple resistance to antibiotics is by pumping them out of the cell, thereby reducing their net uptake (5, 12, 124, 131, 158). This resistance mechanism, which was once confused with increased impermeability, is now known to be executed by several complex efflux systems expressed by the chromosomal genes mexXY, mex EF-oprN, mexAB-oprM, and mexCD-oprj. The susceptibility of all classes of antibiotics to these efflux pumps, with the exception of cationic antimicrobial peptides (CAPs), has been demonstrated (161, 289, 294, 297, 458).

1.2.3. Enzymatic Modification

In addition to the aforementioned resistance mechanisms, *P. aeruginosa* is equipped with enzymes that can modify specific antibiotics or their targets. Some of these enzymes are derived from the expression of chromosomal genes (*e.g.*, the inducible *ampC* gene expressing β-lactamase), or of plasmid-mediated genes (39, 42, 149, 284, 296, 319, 364). In some cases, β-lactamase inhibitors are used clinically to overcome the resistance phenotypes. However, *P. aeruginosa* also develops resistance to β-lactamase inhibitors such as clavulanic acid and tazobactam (15, 279, 283, 285). *P. aeruginosa* may become resistant by acetylation of specific antibiotics or of their targets (98). Furthermore, *P. aeruginosa* may use a rare mechanism of resistance against some cationic antimicrobial peptides (*e.g.*, colistin), which is the replacement

of a phosphate group with a fatty acyl or aminoarabinose group in the sugar portion of lipid A (LPA) (137, 333). These are just a few examples of how this organism modifies its antimicrobial target and becomes resistant to a variety of drugs. Please refer to Lambert (2002) for an extensive review of antibiotic resistance mechanisms of *P. aeruginosa* (268).

1.2.4. *Pseudomonas aeruginosa* Disease

Based on the status of the host immunity, *P. aeruginosa* exhibits a very broad range of clinical manifestations. *P. aeruginosa* may colonize the skin, the lungs, and various other tissues. In some instances, this organism is able to enter the blood (bacteremia), which may lead to sepsis and mortality. This pathogen severely affects patients in intensive care units, burn victims, and patients with cancer, ventilator-associated pneumonia (VAP), AIDS, and CF (7, 16, 17, 31, 32, 37, 38, 41, 51, 54, 57, 60, 85-89, 100, 103, 112, 125, 139, 141-143). The latter is a classical example of the significance of antimicrobial resistance in the clinical management of infectious diseases. CF patients have a genetic disorder in the chloride conductance transmembrane regulator (CFTR), leading to airway-surface-liquid dehydration, coupled with decreased transport and hypersecretion of mucus in response to infections. The mucosal immunity becomes compromised, which results in increased susceptibility to bacterial pathogens. Thus, CF patients require frequent antibiotic treatments to control their chronic bacterial infections and are susceptible to selection for antibiotic resistant isolates. In fact, most case fatalities in CF are associated with infections of the respiratory system, particularly by *P. aeruginosa (92, 106, 114, 115, 119, 120, 122, 128, 129, 151, 157, 170, 171)*. Hence, the occurrence of antibiotic resistance

underscores the need for developing novel antimicrobial agents to complement current antimicrobial regimens.

1.3. Cationic Antimicrobial Peptides

1.3.1. In Search of Novel Antimicrobial Therapeutics

The evolutionary success of multicellular organisms is largely due to the development of very diverse and elaborate defense systems, known collectively as host immunity. The epithelial surfaces of plants and animals are continuously exposed to potentially invasive and deadly pathogens. It is essential that the host immunity provide a mechanism of rapid clearance or inactivation of most of these microbes. An essential and functionally conserved component of the host immunity that fulfills this role is a group of agents known as antimicrobial peptides. The majority of these antibiotics are highly cationic and tend to fold into amphipathic structures (18, 50, 63, 67, 90, 219, 342, 394). Thus, they are often referred to as cationic amphipathic peptides or CAPs (162).

Most of the support for the antimicrobial properties of CAPs has come from intense investigations during the last 20 years. However, the field of antimicrobial peptides began in the late 19th and early 20th centuries with the discovery of complement and several other basic antibacterial tissue factors. Complement, initially termed alexin, was first described as a thermolabile substance that was specific for Gram-negative bacteria and that lost its activity in defibrinated blood. Further studies characterized alexin as a complex system of several components then referred to as the complement (318, 377). With the recent characterization of the antimicrobial properties of C3a, the complement can be now associated with antimicrobial peptides research (317). In 1922, Alexander Fleming reported the isolation of a ‘remarkable

bacteriolytic element' of leukocytic granules derived from the nasal secretion of a patient with acute coryza. He elucidated the antibacterial properties of this heat-stable basic protein, which he termed lysozyme (184). As an enzyme that digests bacterial cell walls, lysozyme is not a typical antimicrobial peptide. However, antibacterial properties of nonenzymatic heat-stable basic proteins with high Arg or Lys contents (*e.g.*, protamine, histones) were also reported (377). During this period of early discoveries, investigators clearly made the association between the cationicity of these proteinaceous agents and their selective interactions with the negatively charged bacterial surface (187). Further, the natural resistance to certain microbial pathogens due to the presence of these basic tissue factors was established (377). In the 1950s and 1960s, Watson, Zeya, and others characterized the interactions between Gram-negative bacterial membranes or nucleic acids and neutrophil lysosomal fractions, which included Arg-rich proteins (75, 76, 448, 451, 453). In 1975, Weiss *et al*. not only distinguished between the activity of a proteinaceous lysosomal fraction and that of lysozyme, but also characterized the electrostatic nature of the peptide interaction with the surface of sensitive bacteria using divalent cations as competitive inhibitors (423). These studies provided the earliest insight on the mechanisms of action of basic antimicrobial peptides. However, it took at least two more decades to elucidate the identity, structural diversity, antimicrobial mechanisms, and the environmental factors affecting the biological activities of these peptides.

By 1980, infectious disease experts were becoming increasingly aware of the problem of antibiotic resistance (1, 2, 4, 97). To address this issue, intensive investigation had been initiated to identify and characterize alternative sources of agents with novel antimicrobial mechanisms. Thus, among the areas of primary focus were the components, functions, and mechanisms of the host immunity of various species (vertebrates and invertebrates). Importantly, the components of

the insect immunity were likely candidates for novel antimicrobial factors, as they had been already described and known to exclude antibody molecules and lymphocytes (180, 240, 335). In insects, a well-characterized mechanism of resistance to infectious pathogens is lysozyme-dependent; however, the restriction of lysozyme efficacy to select gram-positive bacteria led to the prediction of the presence of other host factors that could be active against a wide range of bacteria (82, 183, 249, 259, 261). In the early 1980s, the preliminary observation that the cell-free hemolymph of immunized insects kills bacteria led Boman and colleagues to isolate 2 basic proteins (cecropins A and B). Cecropin peptides were later identified in other species, and their broad activity and selective toxicity toward Gram-negative bacteria characterized (81, 82, 241, 248, 336, 385, 428). During that time, another peptide (mellitin) from bee venom was described as both bactericidal and toxic to red blood cells (408). In contrast, the cecropins did not display significant hemolytic property. The primary sequences of these cationic antimicrobial peptides (CAPs), which displayed high selective toxicity against bacteria, were among the first to be elucidated. A few years later, Dr. Michael Zasloff, while performing experimental surgery on the African frog *Xenopus larvae*, was surprised by the resistance to infections afforded by predicted substances in the frog skin. In 1987, he reported the isolation and activity of magainin 1 and magainin 2 against several organisms. The peptides displayed the highest potency against *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas putida*, and *Staphylococcus epidermidis* (446). An important lesson learned from this study was that the activities of these peptides were dependent on the test organism.

Similar to the studies that led to these aforementioned discoveries, several were initiated on antibacterial properties of neutrophil azurophil granules in mammals, which ultimately resulted in the identification of a group of antimicrobial peptides known as the defensins. In a

series of experiments consisting of lysosomal fractionation, antimicrobial killing assays, protein purification, etc., Gantz, Lehrer, and colleagues isolated the rabbit microbicidal cationic proteins (MCP) and the human neutrophil proteins (HNP), characterized their antibacterial, antiviral, and antifungal activities, and elucidated their primary amino acid sequences. These newly identified peptides were classified as mammalian defensins. These studies were remarkable for bringing to light not only the existence of mammalian CAPs, but also their antifungal, antiparasitic, and antiviral properties (14, 84, 148, 276, 278, 311, 362, 402, 415, 424). Furthermore, Tomas Gantz, using several techniques including enzyme immunoassay, polyacrylamide gel electrophoresis, *etc*., was able to elucidate the secretory mechanism of human neutrophil defensins and quantify the amount of peptide (4 to 5µg/10^6 PMN) released *in vitro* by neutrophils in response to stimuli (*e.g.*, phorbol myristate acetate) (194). The discovery of the cecropins, magainins, and mammalian defensins marked a turning point in the advancement of the field of antimicrobial peptides research.

After hundreds of peptides had already been identified, a newly discovered family of CAPs, the cathelicidins, became (along with the defensins) the subject of intense investigations. Similar to the defensins, the cathelicidins are found in mammals, including human. These gene-encoded peptides are synthesized as inactive precursors consisting of a secretory signal sequence, a highly conserved 5' terminus region (propeptide), and species-specific, highly diverse antimicrobial sequences (Figure 2) (441). The study of these two peptide families (defensins and cathelicidins), coupled with that of engineered and other host-derived peptides, has significantly enhanced the understanding of the secretory and antimicrobial mechanisms of CAPs, the influence of different environments on activity, and their multiple roles in host immunity (30, 147, 193, 201, 295, 328, 378, 398, 411). To date,

more than 1000 natural antimicrobial peptides have been identified, and most (if not all) organisms reportedly use antimicrobial peptides as part of their immune systems.

Figure 2. The Gene structure of a typical antimicrobial peptide (adapted from Zaiou and Gallo, 2002) (439)**.**

1.3.2. Structural Diversity

One of the surprising features of antimicrobial peptides is the conservation of antimicrobial functions despite their structural diversity. Mammalian antimicrobial peptides are generally synthesized as part of a protein with no antimicrobial function. As illustrated in Figure 2, there is a signal sequence that serves to guide the protein to secretory vesicles. The active form of the peptide is released after protease-specific digestion (225, 308, 328, 374, 411). The highly conserved property of a particular family of CAPs is the anionic propeptide sequence, which electrostatically interacts with the antimicrobial domain and inhibits its activity until cleavage occurs by specific proteases (287). The propeptide is sometimes used as a basis for classification. For instance, in the inactive form of mammalian cathelicidins, the propeptide is a highly conserved cathelin (cathepsin protease inhibitor family)-like protein, but the cathelicidins and other CAP families can be further grouped according to their native secondary conformations (α-helix, β-sheet, loop) or amino acid content (58, 63, 72, 95, 272, 274). As demonstrated by structural analyses of CAPs in the presence of liposomes or membrane mimitope solvents (*e.g.*, Trifluoroethanol) using NMR or circular dichroism, the cathelicidins can specifically adopt different types of secondary conformations. For example, the human cathelicidin LL-37 folds

into a α-helix, whereas the porcine protegrins assume β-sheet and loop structures (95, 264, 272, 274, 432). Cathelicidins are also distinguished according to amino acid contents (*e.g.*, the Pro-rich PR-39 and Trp-rich indolicidin) (10, 190, 345). Unlike the cathelicidins, the defensins, a major family of CAPs, tend to adopt only β-sheet conformations. A major reason for this highly conserved native structure in defensins is the presence of 6 Cys residues, which form 3 disulphide bridges that stabilize the β-strands (200, 243, 282, 376).

A common property of antimicrobial peptides is their tendency to fold into amphipathic structures. The diversity of the peptide primary structures is due to the necessity of the host immunity to successfully adapt to different environments by retaining its efficacy against specific microbial pathogens. Hence, the conservation of antimicrobial functions is highly dependent on the amphipathic properties of CAPs and not necessarily on the retention of primary sequence homology. Yet amphipathicity is highly influenced by the amino acid composition and arrangement in the primary sequence, which affects the specificity of antimicrobial activity in a given environment. For instance, the defensins of the human airway have evolved to be optimally active in hypotonic environments. This explains why their antibacterial activity is suppressed in abnormally higher salt concentrations (*e.g.*, the CF airway) (47, 48). Similarly, antimicrobial activities of most host-derived CAPs are markedly inhibited in serum or blood-derived matrices (381). An important concept that has been described to explain structural diversity in the face of conservation of antimicrobial activity is immunorelativity, or immunity in the context of specific environments and invading pathogens. Immunorelativity is the basis for the specific tissue distribution of CAPs in a particular organism. For instance, in humans the predominant antimicrobial peptides of the skin (*e.g.*, LL37, β-defensins. *etc*.) may be different from those of the mucosal surfaces of the oral cavity, the reproductive system, and others (196,

316, 356, 412). These different environments are likely to be exposed to different pathogens and to have different fluid compositions over millions of years of evolution. Thus, the diversity of peptide primary sequences, coupled with the conservation of the amphipathic properties, is mainly dictated by the host environment, including specific pathogens that are likely to be associated with that environment.

1.3.3. Biological Properties

The field of antimicrobial peptides is a highly neglected aspect of immunology in the sense that it is rarely, if ever, addressed in typical immunology textbooks. Ironically, antimicrobial peptides represent the first line of defense against invading pathogens by providing a rapid and effective defense mechanism against potentially harmful pathogens. Although some antimicrobial peptides (*e.g.*, the human neutrophil peptides) play an essential role in circulating neutrophil-mediated microbial killing, most pathogens usually face the mucosal-derived antimicrobial peptides prior to the intervention of circulatory phagocytes (44, 46, 49, 147, 164, 175, 176, 181). While the study of microbial killing mediated by neutrophils and macrophages has primarily brought to light the importance of CAPs in the innate immunity, the discovery of epithelial-specific distribution of antimicrobial peptides indicates a more essential and larger role of mucosal surfaces in host immunity than early reports on antimicrobial peptides might have suggested (46, 242, 329). The interactions of microbial pathogens with local phagocytes or epithelium-derived CAPs are likely to precede the events leading to the activation of circulatory phagocytes and subsequent microbial killing by these phagocytes.

The epithelial surfaces are continuously exposed to microbial pathogens in the environment. The primary task of the host immunity is to prevent the colonization and invasion of the host epithelia by potential pathogens while sparing the organisms that comprise the normal flora. Thus, epithelial cells have the ability to express and secrete antimicrobial peptides either constitutively or in response to microbial presence. The inducible expression of CAPs can be stimulated either by inflammatory cytokines or by the interactions of microbial organisms and endotoxins with the epithelial cells (409). Hence, the human defensins-5, 6 (HD-5, 6) and human β-defensin-1 (hBD-1) are constitutively expressed and secreted in the genitourinary and the gastrointestinal tracts (HD-5, 6 and hBD-1), whereas hBD-2 expression is inducible in the skin, lungs, and several other tissues. Particular noteworthy is the fact that the constitutive expression of a CAP in a specific tissue does not exclude its modulation under certain conditions or in other tissues (135, 322, 323, 337, 338, 375, 382, 412). In epithelial cells, the active forms of CAPs are released into the extracellular milieu after cleavage of the inactive protein by a specific proteolytic enzyme (357). However, a significant portion of antimicrobial peptides localized in phagocytic granules kill their target intracellularly. Similar to the cathelicidins and the defensins of epithelial cells, these peptides are synthesized as inactive proteins. Nevertheless, the post-translational processing leading to the active structures occurs during the myeloid cell differentiation into granulocytes. In the activated phagocytes, the peptides reach their targets by the fusion of the phagocytic granules with the phagolysosomes, although extracellular release may occur (46, 78, 101, 167, 198, 200, 202, 229, 273-275, 277, 321, 328, 363, 427).

A broad spectrum of microbial targets has been identified either by mechanistic studies of non-oxygen mediated microbial killing of neutrophils or by standard *in vitro* microbial killing assays with specific CAPs. Antimicrobial peptide activity of a given CAP may be specific to a

wide range of Gram-negative or -positive bacteria, or both. Other microbial targets may include atypical bacteria, parasites, fungi, and enveloped viruses (197, 443-445). The structural requirements for optimal activity against specific environment-associated pathogens have driven the compartment-specific differentiation of CAPs. Thus, LL37 and the defensins of circulating phagocytes display broad activity against bacteria, fungi, parasites, and enveloped viruses. The defensins of alveolar macrophages or airway epithelial surface are highly potent against pathogens of the airway. Similarly, the activity of CAPs localized to the other mucosal surfaces (reproductive tract, the urinary tract, and others) is likely to be optimal for pathogens associated with those tissues.

The antimicrobial properties of CAPs are demonstrated in the context of the host immunity as a whole. Studies of the best-known human antimicrobial peptide families, the defensins and the cathelicidin, LL37, show that CAPs can promote leukocyte chemotaxis and phagocytosis (*e.g.*, LL37 and α-defensins) (195, 263, 272, 314, 315, 440). For instance, the human β-defensin hBD-2 displays CCR6-mediated chemotactic activities specific to immature dendritic cells and T lymphocytes, while LL37 demonstrates a similar chemotactic activity on T cells, neutrophils, and monocytes by interacting with the formyl-peptide receptor-like 1 (FPRL1) (159, 203, 238, 315, 399, 431). The ability of these peptides to upregulate the level of inflammatory cytokines has been demonstrated *in vitro* and *in vivo* (413, 414). Therefore, it is not surprising that, as mediators of inflammation, they cause tissue injury in the setting of a local infection. Further, these peptides play a series of other roles such as modulating adaptive immunity and wound healing, suppressing hormonal activity and fibrinolysis. For a more extensive discussion of the multiple biological functions of CAPs, please refer to the reviews by Hancock, Lehrer, and others (156, 222, 358).

1.3.4. Mechanisms of Selective Toxicity

An essential property of a safe antimicrobial factor is toxicity against its microbial targets without harming the host. The ubiquity of antimicrobial peptides suggests that their antimicrobial property needs not be associated with host toxicity. However, the specific tissue distribution in a given animal species indicates that host toxicity may also depend on the environment. As previously discussed, the peptides are synthesized as inactive proteins prior to exposure to their targets, and CAP activation by post-translational processing ensures that the cellular membrane structures are protected from any contact with the active peptides prior to reaching their microbial targets. For the peptides that are secreted in specific extracellular milieu, the peptide concentration may be effective against specific pathogens but sublethal to host cells. In addition to environmental influence, there are some fundamental structural properties that may generally render microbial organisms more susceptible than the host to antimicrobial peptides. This antimicrobial selectivity, although not completely understood for several antimicrobial peptides, is partially explained by differences in lipid composition of eukaryotic and bacterial cell membranes (59, 70). Eukaryotic membranes are likely to form hydrophobic (weaker than electrostatic) interactions with CAPs due to the lower proportion of negative lipids and the presence of cholesterol in their membrane outer leaflet. In general, when CAPs (*e.g.*, magainins, cecropins, LL37) are dissolved in aqueous solvents, they demonstrate a disordered structure or random coil as revealed by circular dichroism analysis. On the other hand, in the presence of increasing concentrations of a membrane mimitope solvent or liposomes there is a concomitant increase in the propensity of these peptides to form α-helices (67, 260, 324). Peptides with other types of native structures, such as the defensins (β-sheet) and protegrins (β-sheet and loop), behave similarly (200, 264). Evidence for electrostatic interactions with variably charged

liposomes is also provided by other established methods, including surface plasmon resonance (SPR) spectroscopy or Biacore analysis. Based on these studies, antimicrobial peptides are known to form stronger interactions with bacterial endotoxins and negatively charged surface phospholipids (*e.g.*, LPS, lipotheic acids, phosphatidyl glycerol, etc.) than with zwitterionic and cholesterol-containing liposomes (153, 212, 231, 455).

Similarly, higher negative:zwitterionic phospholipid ratios enhance CAP-induced leakage of fluorescent molecules (*e. g.*, calcein) from model membranes (105, 123, 207, 299, 300, 456). Thus, electrostatic interactions between CAPs and negative lipids on the bacterial surface may provide the energy that drives CAP insertion in microbial membranes. Another key modulator of CAP-membrane interactions is hydrophobicity. While cationicity is the primary determinant of antibacterial selectivity, hydrophobicity probably enhances activity against Gram-positive organisms and may compromise antimicrobial selectivity if raised beyond an optimal level. Hence, the amphipathic nature of CAPs, or the combined structural contributions of hydrophobicity and cationicity are critical to antibacterial selectivity (154, 155, 206, 217, 262). What remains to be established, however, is whether the strength of this initial peptide-membrane interaction correlates with antimicrobial activity.

An area of controversy is the mechanism of microbial killing. There are several models of the killing mechanism, each supported by the study of specific groups of peptides. One of the earliest approaches consists of biochemical analyses of membrane permeabilization by cationic peptides. In these studies, bacteria are exposed to peptides in the presence of an impermeable substrate (ONPG) for the intracellular enzyme β-galactosidase. The increased activity of this enzyme as a function of time or peptide concentration is indicative of cytoplasmic membrane

permeabilization. Fluorescence spectroscopy studies using peptide-mediated 1-N-phenylnapthylamine (NPN) uptake (indicating outer membrane permeabilization) or calcein released from variably charged artificial membrane further support the formation of pores leading to leakage of cellular content (207, 266, 271, 298, 299, 456). These studies help to establish the ability of some peptides to permeabilize either the outer membrane only (*e.g.*, polymyxin B) or both outer and inner bacterial membranes (*e.g.*, lentiviral lytic peptide 1 or LLP-1) (331). Consistent with the biochemical and fluorescent analyses is the observation of bacterial membrane damage (disorganization, blebbing, etc.) by electron microscopy. These studies led to the partial evaluation of the antimicrobial mechanism of the pore-forming peptides. However, to elucidate the steps involved in membrane lysis, the use of other methods is necessary.

With advanced structural analyses using oriented CD, NMR, and X-ray crystallography among other techniques, investigators are able to determine the important steps involved in membrane penetration. Three models for lytic mechanisms have been distinguished. In the barrel-stave model, some amphipathic α-helical peptides may perpendicularly penetrate the lipid bilayer with the hydrophilic regions of aggregated molecules facing the center of the pore and the hydrophobic domains directly in contact with the fatty-acyl groups (lipid core of the bilayer) (172, 269, 270, 383, 433). Yet some other helical peptides may accumulate on the surface of the bilayer in a head-to-tail fashion by lying in parallel to the outer and inner leaflets, leading to formation of micelles as observed for detergents. This “carpet-like” bacterial killing is mainly attributed to the antimicrobial peptide ovispirin, an 18-mer CAP with broad-spectrum antibacterial activity (366, 367, 429, 430). A third mechanism, the toroidal model, shares some common features with the other two. It is similar to the barrel mechanism by the perpendicular

insertion of the peptide leading to the formation of pores but differs from this model by the continuous association of the peptide with the lipid head groups. Similar to the carpet model, the inner lining of the pore consists of the peptide and the associated lipid head groups. Examples of CAPs inducing this type of membrane pore are provided by the magainins (frog skin), protegrins (porcine cathelicidin), and mellitin (antibacterial and hemolytic CAP from bee venom) (19, 290, 379, 433).

A different view on the mechanism of action of CAPs involves the penetration of the cell followed by electrostatic interactions with the negatively charged DNA and RNA. Support for this type of model comes from studies of buforin II (histone-derived peptide), indolicidin, and other peptides, which indicate that microbial killing occurs by inhibition of the major vital intracellular processes (*e.g.*, DNA replication), sometimes coupled with the absence of pore formation. This alternative view is strongly supported by the identification of a hinge region in Buforin II, which promotes cellular penetration of chimeric CAPs consisting of the hinge sequence and a pore forming sequence (179, 310, 313, 326, 334, 388, 390).

1.3.5. Engineered Antimicrobial Peptides and Potential Applications

One of the most important lessons we have learned from the studies of host-derived peptides is that antimicrobial activity and selective toxicity essentially depend on amphipathicity and environmental conditions. The diversity of CAPs, dictated by the necessity for optimal antimicrobial activity in a given environment, indicates that the engineering of a peptide with universal applications is highly unlikely. Based on this understanding, several attempts have been made to develop CAPs for specific applications. Hence, magainin derivatives were first

generated to treat diabetic foot ulcer (205, 210). Unfortunately, this project prematurely ended because the approval for clinical use was not granted by the Food and Drug Administration (FDA). However, the polymyxins have been used clinically against *P. aeruginosa* in severe cases of gram-negative bacterial sepsis. The latest polymyxin derivative, colistin, is used either IV or by inhalation in the treatment of respiratory infections caused by *P. aeruginosa* in CF patients, but there is major toxicity (particularly nephrotoxicity) associated with its systemic use (65). Derivatives of histatins, a family of salivary antimicrobial peptides, have been successfully engineered and tested for the treatment of oral infections (346). In most cases, the development of CAPs for topical applications is dictated by the suppression of activity in biological fluids (*e.g*., blood, plasma, or serum). The potential of antimicrobial peptides for clinical applications is yet to be fully explored.

Based on this understanding, we hypothesized that c*ationic antimicrobial peptides (CAPs) with optimized amphipathic structures could be engineered* de novo *to enhance antibacterial activity and selective toxicity in environments that are generally challenging to host-derived peptides*. Furthermore, *antibacterial efficacy would positively correlate with length, helicity, charge, and Trp content.*

1.4. Specific Aims

1.4.1. Specific Aim 1

To design *de novo* engineered cationic amphipathic peptides (eCAPs) and evaluate their antimicrobial activity. We evaluated the functional properties of a series of *de novo* engineered CAPs that contain multimers (2, 3, & 4 repeats) of a 12-mer lytic base unit (LBU) composed only of Arg and Val residues or multimers of a modified unit (WLBU) that contains specific Trp substitutions, both arranged to form an idealized amphipathic α-

helix. Peptide antimicrobial activities were correlated with length, helicity, and Trp content to establish the structural determinants of antimicrobial activity.

1.4.2. Specific Aim 2

To examine the mammalian cytotoxicity and sensitivity of select peptides to varying salt concentrations and biological fluids by bacterial killing assays (BKA). Initial characterizations of CAP cytotoxicity in our lab and others have been based on human erythrocyte lysis assays (371, 396). While easy to perform, the relevance of these assays to host-pathogen interactions in biological fluids and specific infections remains uncertain. Therefore, we have developed a series of novel selective toxicity assays by measuring the selective toxicity of candidate peptides in a mixed culture of bacteria and human cells. In these assays, bacterial and host cell viability are monitored as a function of peptide added to assess CAP selective toxicity.

1.4.3. Specific Aim 3

To evaluate the antibacterial efficacy of select peptides in experimental mouse model of *P. aeruginosa* bacteremia. To complement our *in vitro* studies, antimicrobial effects of our lead peptide were evaluated in two acute infection mouse models. In the intraperitoneal (IP) (245) model, the peptide WLBU2 was administered intravenously 1h after IP injection of bacteria. An intravenous (IV) infection model was then developed to further examine the efficacy of WLBU2. In this model, the prophylactic potential and the therapeutic effects of the peptide were determined in a dose-dependent manner. Outcomes were evaluated and compared by monitoring mouse survival over time, the level of bacterial cfu per mL blood

or per gram of tissue (liver, lungs, and spleen), and the level of inflammation by histopathology and ELISA for assessment of cytokine profiles in isolated serum samples. These novel animal models provide important information on the *in vivo* potential of WLBU2 and serve as a basis for consideration of human clinical trials to treat *P. aeruginosa* septicemia.

The following two chapters are published articles with minor modification to formatting used with permission of ASM Press:

1. Deslouches B, Phadke SM, Lazarevic V, Cascio M, Islam K, Montelaro RC, Mietzner TA. *De novo* generation of cationic antimicrobial peptides: influence of length and tryptophan substitution on antimicrobial activity. Antimicrob Agents Chemother. 2005 Jan;49(1):316-22.

2. Deslouches B, Islam K, Craigo JK, Paranjape SM, Montelaro RC, Mietzner TA. Activity of the de novo engineered antimicrobial peptide WLBU2 against Pseudomonas aeruginosa in human serum and whole blood: implications for systemic applications. Antimicrob Agents Chemother. 2005 Aug;49(8):3208-16.

2. CHAPTER 2: DE NOVO GENERATION OF CATIONIC ANTIMICROBIAL PEPTIDES: INFLUENCE OF LENGTH AND TRYPTOPHAN SUBSTITUTION ON ANTIMICROBIAL ACTIVITY

2.1. Abstract

Studies of lentiviral lytic peptide 1 (LLP1) of HIV-1 transmembrane glycoprotein gp41 and other host-derived peptides indicate that antimicrobial properties of membrane-active peptides are markedly influenced by their cationic, hydrophobic, and amphipathic properties. Many common themes, such as Arg composition of the cationic face of an amphipathic helix and the importance of maintaining a hydrophobic face have been deduced from these observations. These studies suggest that a peptide with these structural properties can be derived *de novo* using only a few strategically positioned amino acids. However, the effects of length and helicity on antimicrobial activity and selectivity have not been objectively evaluated in the context of this motif. To address these structure-function issues, multimers of a 12-residue lytic base unit (LBU) peptide composed only of Arg and Val residues aligned to form idealized amphipathic helices were designed. Bacterial killing assays and circular dichroism analyses revealed a strong correlation between antibacterial activity, peptide length, and the propensity to form α-helices in solvents mimicking the environment of a membrane. Increasing peptide length beyond two LBUs (24-residue peptides) resulted in no appreciable increase in antimicrobial activity. Derivatives (WLBU) of the LBU series were further engineered by substituting Trp residues in the hydrophobic domains. The 24-residue WLBU2 peptide was active at physiologic NaCl concentrations against *Staphylococcus aureus* and mucoid and non-mucoid strains of *Pseudomonas aeruginosa*. Further, WLBU2 displayed the highest antibacterial activity of all peptides evaluated in this study using a co-culture model of *P. aeruginosa*

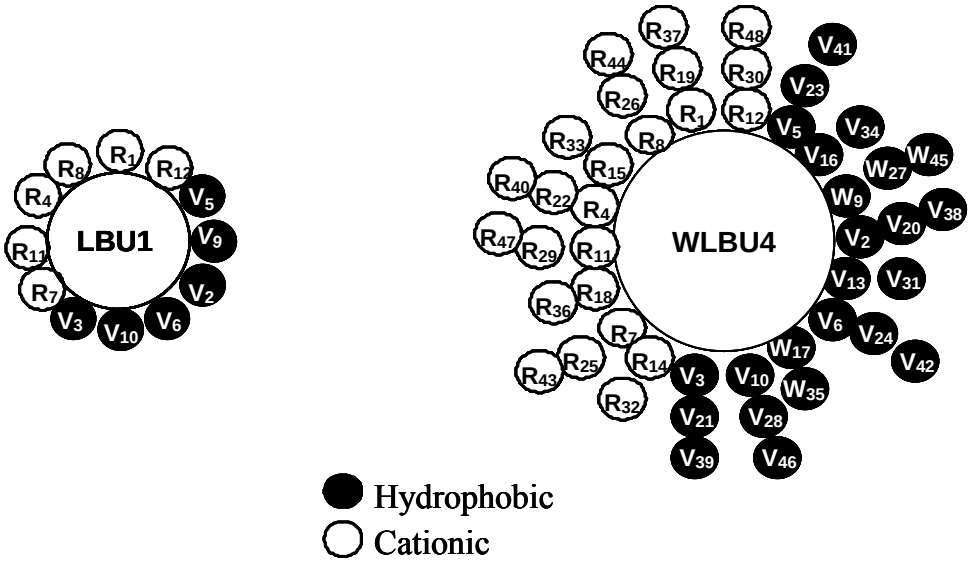
and primary human skin fibroblasts. These findings provide fundamental information toward the *de novo* design of an antimicrobial peptide useful for the management of infectious diseases.

2.2. Introduction

Despite the establishment of safe and effective antibiotics, the management of infectious diseases remains a worldwide public health concern. One untapped resource of novel antimicrobial agents, evolutionarily proven as anti-infectives, are the host derived antimicrobial peptides (218, 221). In vertebrates, insects, and even bacteria, these compounds represent the first line of defense against pathogens challenging the host (221). These peptides commonly adopt an amphipathic conformation in which positively charged and hydrophobic groups segregate onto opposing faces of α-helices, ß-sheets, or other tertiary structures (343). These general structural properties confer an ability to disrupt or traverse a phospholipid membrane. The spectrum of different amino acid side chain chemical properties affords a variety of peptide sequences to present a cationic amphipathic helical peptide. As a result, hundreds of different natural cationic antimicrobial peptides (CAPs) with widely varying sequences have been described. The diversity of antimicrobial peptides is likely a consequence of each peptide evolving to function in a particular environment against a specific subset of microbial pathogens.

We have previously reported the conservation of a peptide derived from the extreme C-terminus of the HIV-1 transmembrane protein that is referred to as the lentivirus lytic peptide 1 (LLP1) (302). Like other membrane interactive peptides, LLP1 is predicted to have an amphipathic α-helical structure and may be involved in calmodulin binding (303, 397, 438) and natural endogenous reverse transcriptase (NERT) activities of HIV-1 (454). Investigations of the

A



B

Peptide	Sequence	*MW (D)
LBU-1	RVVRVVRRV VRR	1,549
WLBU-1	RVVRVVRRWVRR	1,636
LBU-2	RR VVRRVRRV VRRVVRVVRRV VRR	3,138
WLBU-2	RRWVRRVRRWVRRVVRVVRRWVRR	3,398
LBU-3	VRRV VRRVVRVVRRV VRRVRRV VRRVVRVVRRV VRR	4,612
WLBU-3	VRRVWRRVVRVVRRWVRRVRRWVRRVVRVVRRWVRR	4,961
LBU-4	RVVRVVRRVV RRVRRV VRRVVRVVRRV VRRVRRVV RRVVRVVRRVVRR	6,145
WLBU-4	RVVRVVRRWVRRVRRVWRRVVRVVRRWVRRVRRWVRRVVRVVRRWVRR	6,579

* Molecular weight in Daltons

Figure 3. Peptide design

The cationic amphipathic peptides were designed as demonstrated in the helical wheel diagrams (A). Arg, Val, and Trp residues were arranged to form idealized amphipathic α-helices, with the hydrophilic and hydrophobic faces indicated in clear and shaded circles, respectively. The 12- and 48-mers LBU1 and WLBU4 are shown as representatives of the LBU and WLBU series, respectively. (B) Primary sequences of the LBU and WLBU peptides used in this study are shown. The shortest peptide forms one lytic base unit (LBU1) of 12 amino acids, and the others were designed as multimers (2, 3, & 4) of LBU1. The WLBU peptides were derived from the LBU series by substituting Trp residues at the indicated positions. Not shown here are the peptide hydrophobic moments calculated according to Eisenberg.

antimicrobial potential of LLP1 demonstrate that the parent sequence is remarkably potent when compared with other host-derived antimicrobial peptides (396). Based on these studies, CAP derivatives can be engineered for enhanced potency and selectivity by increasing cationicity using Arg residues on the polar face and hydrophobicity using Val residues on the non-polar face (395). This potency and selectivity can be further enhanced by increasing peptide length and by including Trp residues on the non-polar face as demonstrated by Vogel and colleagues (417).

Similar to studies by McLaughlin and colleagues (251) and DeGrado and colleagues (400), we reasoned that a *de novo* peptide presenting an optimized amphipathic α-helix with exclusively Arg residues on the hydrophilic face and Val residues on the hydrophobic face would demonstrate antimicrobial activity, as shown in this report. An important question requiring attention was the optimal length of the peptide and the influence of Trp residues on the hydrophobic face. In this study we describe the design and evaluation of *de novo* engineered peptides of varying length and Trp content for their *in vitro* activity and selectivity in a bacterial:host cell co-culture model. The results demonstrate that it is possible to engineer an effective antimicrobial compound using a 24-residue peptide comprised only of Arg and Val residues. Notably, increasing peptide length did not enhance antimicrobial potency *in vitro*, and inclusion of Trp residues on the hydrophobic face conferred increased selectivity in the bacterial:host cell co-culture model.

2.3. MATERIALS AND METHODS

2.3.1. Peptide Synthesis

The LBU and WLBU series (Figure 3), as well as the host-derived antimicrobial peptide LL37 (191, 406) were synthesized using standard Fmoc synthesis protocols as previously described (185). Synthetic peptides were characterized and purified by reverse-phase HPLC on Vydac C18 or C4 columns (The Separations Group, Hesperia, CA), and the identity of each established by mass spectrometry (Electrospray Quatro II triple quadruple mass spectrometer, Micromass Inc., Manchester, UK). Peptide concentrations were determined using a quantitative ninhydrin assay as previously described (185).

2.3.2. Bacterial Killing Assays

Bacterial killing assays were conducted as previously described (396) using two prototypical pathogens, a clinical isolate of *Pseudomonas aeruginosa* (PA1244) and a laboratory-passaged strain of *Staphylococcus aureus* (396). Each of these isolates was confirmed to react identically to biochemical tests as American Type Culture derived strains of *P. aeruginosa* and *S. aureus* and was demonstrated to be susceptible to all antibiotics tested. Also used for this study were two mucoid *P. aeruginosa* strains purchased from American Type Culture Collection and a series of clinical isolates identified by Children's Hospital of Pittsburgh as *P. aeruginosa* exhibiting either a mucoid or a non-mucoid phenotype.

To test for susceptibility of these index bacteria to the peptides described, bacterial suspensions (*ca.* 1×10^6 cfu/mL) in 10mM potassium phosphate buffer (PB) or phosphate buffer containing 150mM NaCl (PBS), pH 7.2, were incubated with two-fold dilutions of peptides for

30 min at 37°C. Serial peptide dilutions were performed and plated on tryptic soy agar (TSA, Difco, Detroit, MI). Surviving colonies were counted the next day to determine the minimum bactericidal concentration (MBC), defined as the molar concentration of peptide reducing the viable bacteria within a suspension by three orders of magnitude. Values were expressed on a molar basis, with a lower MBC corresponding to increased peptide potency. The results were expressed as an average of MBC values obtained from three to five independent experiments. MBCs may be converted to micrograms per milliliter by dividing molecular mass (in g/mol) by 1000 and then multiplying by the value in micromolar units.

2.3.3. Circular Dichroism Analysis

Circular dichroism (CD) was performed as previously described (185). Briefly, CD measurements were taken at 25ºC with an Aviv spectrometer (Aviv Instruments, Lakewood, NJ), over the range of 190-300 nm, and in PB or 30% trifluoroethanol (TFE) in PB. The average mean residue ellipticities ([Θ]/1000 x degree x cm2/dmol) for 8-10 scans per experimental trial were plotted against wavelengths (nm), and the program K2D (http://www.embl-heidelberg.de/~andrade/k2d/) used to determine the percent helicity for each peptide in TFE (28).

2.3.4. Selective Toxicity

Primary human skin fibroblast (HSF) cells at passage 20 were propagated at 37ºC and in 5% CO_2 to over 80% confluence in Iscove's Modified Dulbecco's medium (IMDM) (Life Technologies, Grand Island, NY) containing 10% bovine fetal serum (BFS) and transferred (after trypsinization) to each well of a 96-well plate to a final count of 0.5 x 10^5 cells/well. After a 24-h incubation at 37ºC, the medium was aspirated and a 100µL suspension of *P. aeruginosa* 1244

(~10^6 cells/ml in 50% serum-free IMDM) was added to each well. Using peptide concentrations from 0 to 10μM in 1x PBS, the bacterial/HSF media were further diluted to 25% IMDM. The co-culture was then incubated at 37ºC for 1h. To determine bacterial survival, the co-culture medium was serially diluted after gentle pipetting to 1:1000 by transferring 20μL aliquots to another 96-well plate containing 180μL PBS per well. Each dilution was plated (100μL) on tryptic soy agar, incubated overnight at 37°C, and bacterial counts determined and expressed as colony forming units per mL (cfu/mL).

Measurements of HSF cell viability were accomplished using a tetrazolium-based colorimetric assay (113, 204, 325). After washing twice with PBS, the co-culture medium was replaced with 100μL IMDM containing 10% FBS (vol/vol) and 0.5 mg/mL MTT Formazan (MTT) (Sigma, Lakehood, NJ). The reaction mixtures were incubated at 37^{o}C in 5% CO_2 for 4h after which equal volumes of 0.1N HCl:isopropanol were added. The percent viability was assessed by taking absorbances at 570nm using a Dynatech MR5000 (Germantown, MD). As controls, cells were treated with 25% IMDM (the test medium) in the presence or absence of bacteria, without the addition of peptide and with 100% lysis buffer [0.15M NaCl, 25mM Tris:HCl (pH 8.0), 1% (g/vol) deoxycholate and Triton X 100]. The experiments were performed in triplicate and viability data averaged. The final HSF toxicity values are expressed as mean percent toxicity for each test condition minus the percent toxicity in the presence of bacteria alone.

2.4. RESULTS

Studies of the conservation of Arg and Val residues in the LLP1 sequences among diverse HIV1 isolates (396), coupled with engineering of the LLP1 sequence for greater potency

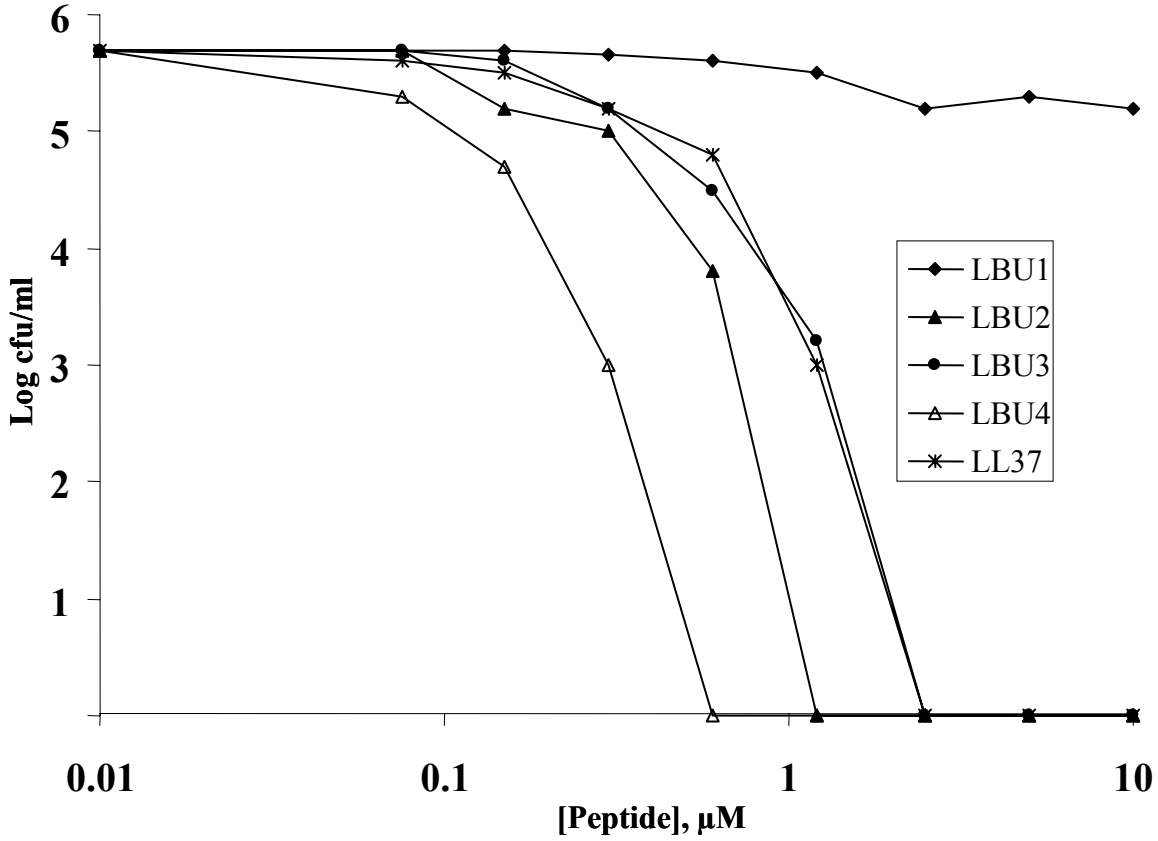


Figure 4. Relationship between length and antibacterial activity

Isolates of *P. aeruginosa* (PA1244) were incubated with two-fold serially diluted peptides at 37°C for 30 min. Bacterial survival at corresponding peptide concentrations was evaluated by broth dilution assays. The activity of the LBU series against *P. aeruginosa* in 10 mM phosphate buffer (PB) is both length- and dose-dependent, reaching almost an optimal level at 24 residues in length.

(361, 395, 396, 407) and other studies related to host-derived antimicrobial peptides (251, 361, 407), led us to propose that effective *de novo* antimicrobial peptides could be synthesized solely from Arg and Val residues if positioned to optimize an amphipathic α-helix. What was not clear

from previous studies was the minimum peptide length required for optimal antimicrobial activity. In addition, since the membrane perturbation properties of Trp-rich antimicrobials (354, 417) and fusogenic peptides (355) have been well described, we hypothesized that the inclusion of Trp residues on the hydrophobic face of an α -helix would increase antimicrobial potency. To address both of these issues, the series of peptides described in Figure 3 was designed based on the concept of a repeating lytic base unit of 12 residues.

2.4.1. Antibacterial Potency of LBU Peptides of Increasing Length

Using a standard broth dilution assay (396), the potency (*i.e.*, antimicrobial activity on a per mole basis) of the LBU series and the host-derived human cathelicidin, LL37, was compared for killing *P. aeruginosa* in PB alone. Figure 4 shows the dose-dependent bacterial survival in Log cfu/mL after peptide treatment. There was a significant increase in activity from the 12-residue LBU1, which did not achieve an MBC at the concentrations tested, to the 24-residue LBU2, which demonstrated an MBC of 0.6μM (Table 1). There was no appreciable increase in potency against *P. aeruginosa* for the 36-residue LBU3 and 48-residue LBU4 peptides. Interestingly, the LBU2 and LBU4 were more active than the host-derived human cathelicidin, demonstrating the increased potency of the *de novo* peptide component to a reference natural antimicrobial peptide.

To define the minimum length for bacterial killing, a series of LBU peptides ranging from 12 to 24 residues were tested for their antimicrobial potency in PB and PBS against two index organisms, *P. aeruginosa* and *S. aureus*. The results (Table 1) indicate that at a length of 24 residues, optimal peptide activity (MBC 0.6μM) was achieved for *P. aeruginosa* in both PB and PBS, although some antimicrobial activity was observed with the 15- and 18-residue derivatives.

Table 1. Influence of length and salinity on antibacterial activity [a]

Peptides	Length (residues)	*P. aeruginosa* PA1244		*S. aureus*	
		MBC (μM)			
		PB	PBS	PB	PBS
LL37	37	1.2	1.2	2.5	>10
LBU1	12	>10	>10	>10	>10
LBU2	24	0.6	0.6	1.2	>10
LBU3	36	1.2	0.6	0.6	1.2
LBU4	48	0.3	0.3	0.3	0.3
WLBU1	12	0.6	>10	>10	>10
WLBU2	24	0.3	0.3	0.3	0.3
WLBU3	36	2.5	0.6	2.5	0.3
WLBU4	48	2.5	0.6	2.5	0.6
LBU1.3	15	1.2	>10	>10	>10
LBU1.6	18	1.2	>10	2.5	>10
LBU1.9	21	0.6	2.5	1.2	>10

[a]The MBCs (peptide concentrations promoting 99.9% killing) of the LBU and WLBU series in PB and PB + 150 mM NaCl (PBS) were derived from the dose-dependent survival curves as previously described (26). (A) The human cathelicidin LL37 was used as a comparative host-derived peptide in these experiments. There was no significant change in antibacterial activity in peptides of 24-48 residues in length, but the peptide LBU2 was salt sensitive against *S. aureus*. (B) The relationship between length and activity was further characterized using peptides of 12, 15, 18, 21, and 24 residues. The results of these studies reveal that a minimum length of 15 residues is required for significant antibacterial activity. Average MBCs were obtained from 3-5 experimental trials.

2.4.2. Influence of Trp residues on Antimicrobial Potency of the LBU peptides

Trp is known to be a highly membrane active amino acid and is prevalent in many antimicrobial peptide sequences (417). The contribution of Trp residues to antimicrobial activity, when included on the hydrophobic side of the LBU peptide series, was investigated (Figure 3). As summarized in Table 1, a single Trp substitution in WLBU1 significantly increased anti-pseudomonal potency when compared to the parent peptide LBU1 in PB, with an MBC of

0.6μM. The 24-residue WLBU2 derivative (three Trp residues in the hydrophobic face) displayed a slight increase in activity against *P. aeruginosa* in comparison to the LBU2, but it showed a significantly higher potency against *S. aureus* with an average MBC of 0.3μM in both PB and PBS. These findings suggest that the inclusion of Trp residues can render peptides less salt-sensitive and significantly more potent against *S. aureus*. As observed for the LBU series, in creased length did not result in increased potency for longer WLBU peptides, although WLBU3 and WLBU4 peptides displayed similar resistance to NaCl for both index organisms.

2.4.3. Relationship of Helicity in Membrane Mimetic Solvents to Antimicrobial Activity

The relationship between the propensity to form a helix and antibacterial activity was investigated using circular dichroism. The LBU peptides described in Figure 3 were subjected to structural analysis in PB (mimicking an aqueous environment) and PB containing 30% TFE (mimicking a membrane environment). Not surprisingly, no appreciable structure was observed for all peptides in PB (data not shown). In contrast, the helical propensity in membrane mimetic solvents did correlate with bacterial killing (Figure 5). In general, a percent helical value of greater than 80% correlated with the potency of bacterial killing (Table 2). This finding is significant for the design of future *de novo* antimicrobial peptides, suggesting that helical content in a membrane environment should be preserved for maximal antimicrobial potency.

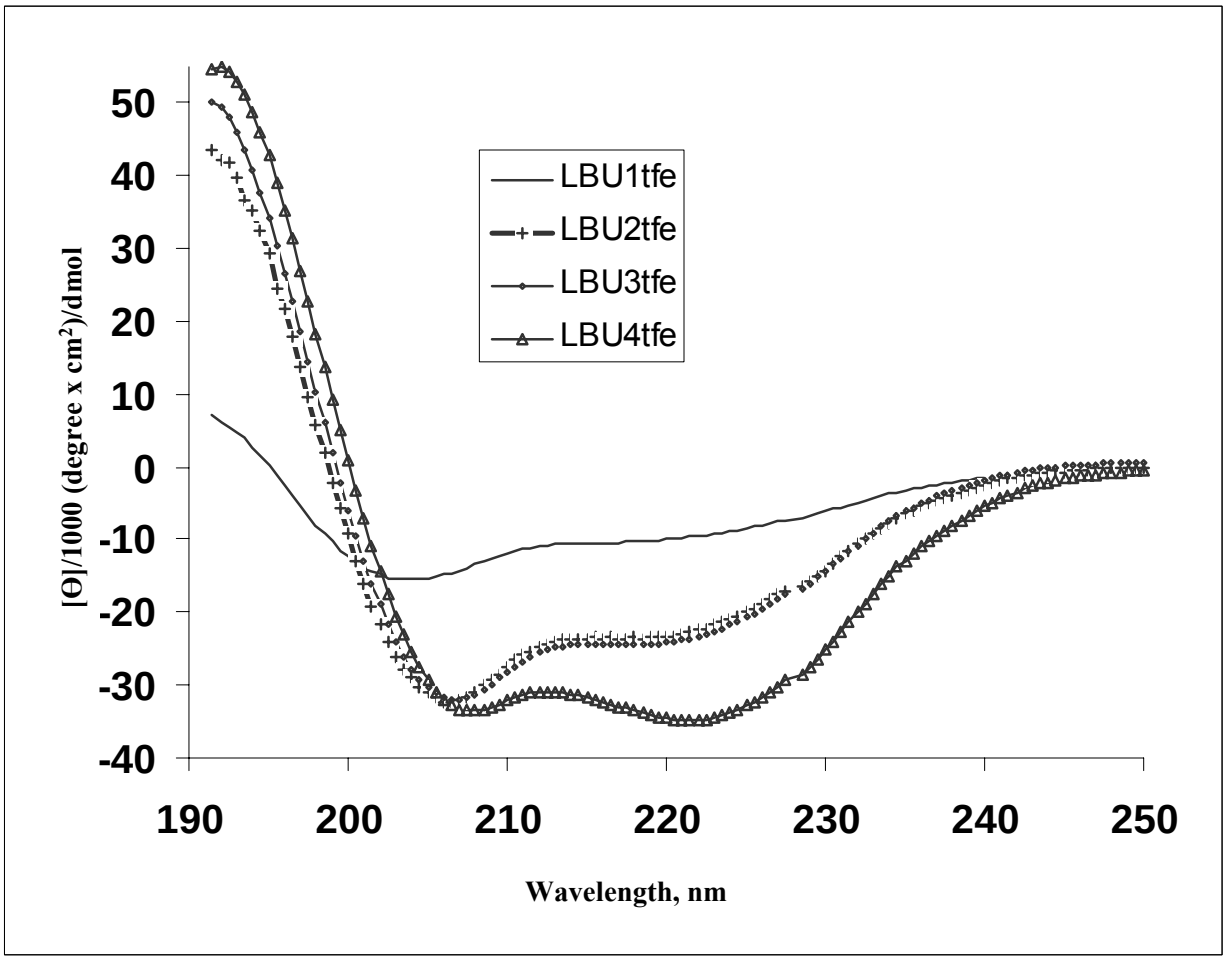


Figure 5. Structure of *de novo* engineered antimicrobial peptides

Circular dichroism analysis was performed on the LBU and the WLBU peptides under two conditions: (not shown) 10 mM phosphate buffer (PB) and 30% trifluoroethanol (TFE) as described previously. Mean residue ellipticities {[Θ]/1000 (degree x cm2)/dmol)} were plotted against wavelength, and the CD spectra shown here are representative of 3 independent experimental trials.

2.4.4. Activity of WLBU2 against a Battery of *P. Aeruginosa* Isolates

Based on the above experimental results, WLBU2 demonstrated the optimal peptide activity, *i.e.*, it was the shortest, most potent peptide active against *P. aeruginosa* and *S. aureus* under physiological NaCl conditions. To investigate whether the potent activity displayed by WLBU2 would be observed against other organisms in addition to the index strain, standard bacterial killing assays were performed in PBS using 16 clinical isolates of *P. aeruginosa*, six of

which were of a mucoid phenotype. The results are reported in Table 3 and demonstrate that WLBU2 maintains potent activity against all strains tested but also displayed a 2-16 fold higher (depending on the strain) bactericidal activity than that of LL37.

Table 2. Relationship between length and helical content [a]

Peptides	Length (residues)	% helicity
LBU1	12	27
LBU2	24	82
LBU3	36	80
LBU4	48	92
WLBU1	12	25
WLBU2	24	81
WLBU3	36	92
WLBU4	48	75
LBU1.3	15	42
LBU1.6	18	45
LBU1.9	21	82

[a]To evaluate the relationship between length and helical content, percent helicity was obtained from the CD data using the program K2d over the internet (www.embl-heidelberg.de/~andrade/k2d/). Although length seems to correlate with helicity, there is little increase in helical content in peptides longer than 24 residues. These data, together with the results of the bacterial killing assays, reveal a similar correlation between helicity and antibacterial activity in phosphate buffer. The data tabulated are representative of 2-3 experimental trials.

2.4.5. Selective Toxicity

Initial characterization of CAP cytotoxicity for human cells in our lab and others have been based on the human erythrocyte lysis assay (146, 265, 396, 401). The results obtained by red

Table 3. Antibacterial activity of WLBU2 is not strain specific [a]

P. aeruginosa Strains	MBC (μM)	
	WLBU2	LL37
PA1244	0.3	2
PACO1*	0.5	2
PACO3*	0.3	1
PACO4	0.5	2
PACO6	0.5	2
PACO7*	0.5	2
PACO8	0.3	1
PACO9	0.3	1
PACO10	0.3	2
PACO11	0.5	2
PACO12	0.5	2
PACO13*	0.5	2
PACO14	0.3	2
ATCC19142*	0.3	5
ATCC33468*	0.3	2
PAO1	0.5	1

*Mucoid type

[a]To investigate whether the potent activity displayed by WLBU2 was dependent on the specificity of the clinical strains, we performed standard bacterial killing assays (in PBS) using a panel of 16 strains of *P. aeruginosa*, six of which were of mucoid phenotype. The peptide not only maintained its potent effects against all these strains, but also demonstrated a 4-13 fold higher bactericidal activity than that of LL37. The data tabulated are representative of 2 experimental trials.

blood cell lysis, while informative, do not reflect the challenges of antimicrobial peptides that must be selectively toxic for bacterial cells over eukaryotic cells presented at the identical time and under identical conditions. To more accurately assess the concept of selective toxicity, we

have developed a co-culture assay in which primary human skin fibroblasts (HSF) are infected with *P. aeruginosa* prior to peptide treatment. After 1h of exposure to peptide, these co-cultures were examined for bacterial killing and eukaryotic cell viability as described in the methods. LBU2 and WLBU2 were chosen for study in this assay because they were the minimal length peptides that demonstrated potent antimicrobial activity (Table 1) and low hemolytic activity (data not shown). Figure 6 plots increasing concentrations of LBU2 (panel A) and WLBU2 (panel B) as a function of bacterial killing (left axis) and eukaryotic cell toxicity (right axis). The cytotoxic effects of LBU2 and WLBU2 on primary HSF cells were negligible at concentrations (<8μM and <2μM, respectively) that decrease bacterial load by 99.9%. These data suggest the inclusion of Trp residues can significantly increase antimicrobial potency in a selective toxicity setting. By comparison, the host-derived LL37 was moderately toxic to HSF cells (31% reduction at 10□M), and a 99.9% reduction in *P. aeruginosa* was not attained at the maximum concentration tested (Figure 4, panel C). The finding on the increased potency of the *de novo* designed antimicrobial peptides when compared to LL37 suggests that peptides of improved potency and selectivity can be generated.

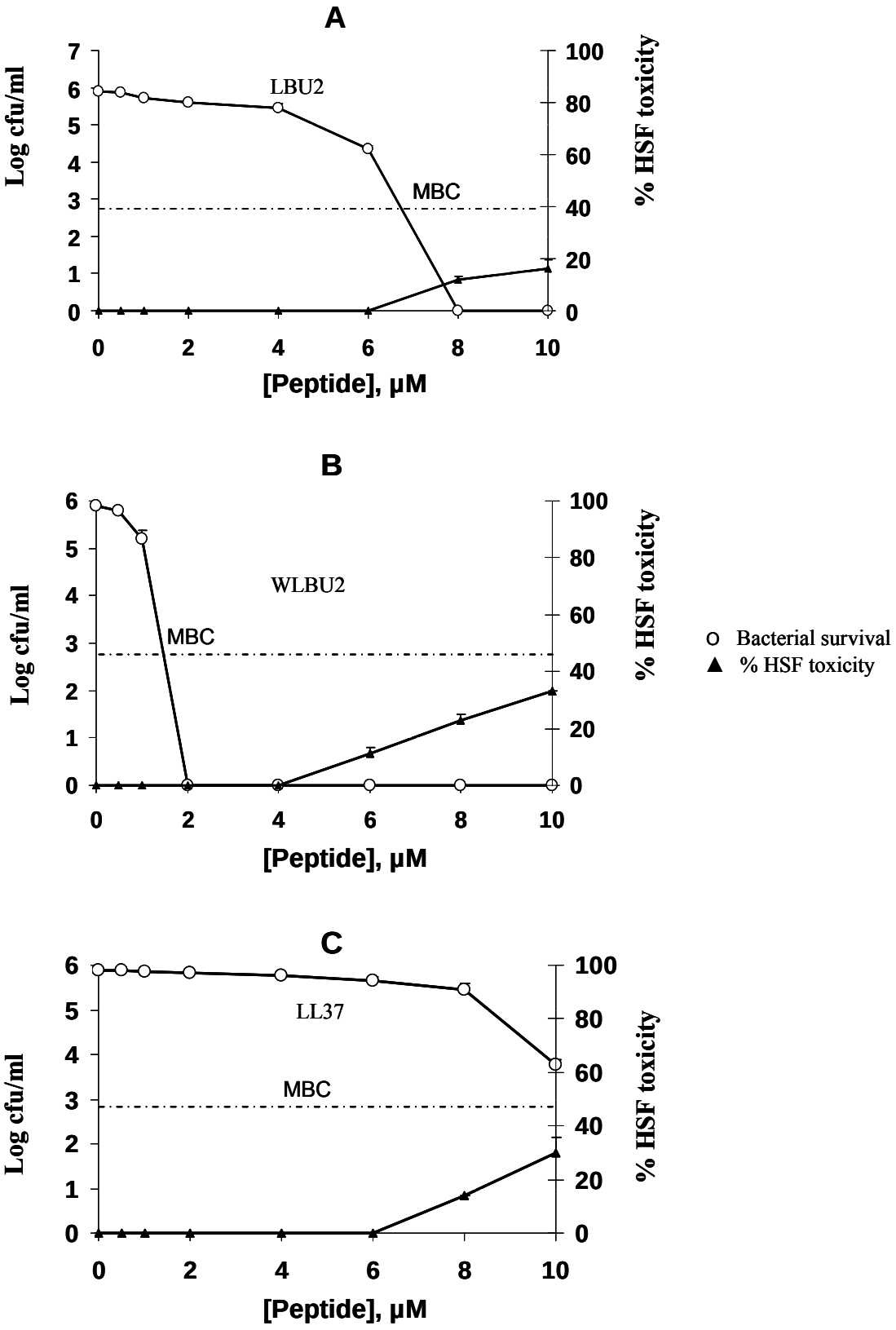

A
LBU2
MBC
Log cfu/ml
% HSF toxicity
[Peptide], μM
B
WLBU2
MBC
Log cfu/ml
% HSF toxicity
[Peptide], μM
C
LL37
MBC
Log cfu/ml
% HSF toxicity
[Peptide], μM
Bacterial survival
% HSF toxicity

Figure 6. Selective toxicity of antimicrobial peptides in a co-culture system

Primary human skin fibroblasts (HSFCCD-986sk) at passage 20 and over 80% confluence were infected with *P. aeruginosa* in 50% Iscove's modified Dulbecco's medium (IMDM) in PBS with no bovine fetal serum. The co-culture was then treated for 1h with 2-fold dilutions of peptides as described in Materials and Methods. Bacterial survival was determined as in standard broth dilution assay, and HSF viability evaluated by MTT staining (a tetrazolium-based assay that measures the activity of mitochondrial redox enzymes). Controls included were 25% IMDM in PBS (0% cytotoxicity), 25% IMDM + bacteria (10^5-10^6 cells/ml), and 1x standard lysis buffer (100% cytotoxicity). The selected peptides LBU2 (A) and WLBU2 (B) displayed high antibacterial potency and selectivity in comparison to the host-derived peptide LL37 (13). Data plotted are representative of three experimental trials.

2.5. DISCUSSION

In this study we demonstrated that antimicrobial peptides can be designed based on a cationic amphipathic motif using Arg residues on the cationic face and Val residues on the hydrophobic face. Furthermore, we demonstrated that increased potency can be achieved by the inclusion of Trp residues on the hydrophobic face (predicted based upon the predominance of these residues in the indolicidins) (384). The current study identifies LBU2 and WLBU2 as lead compounds for *de novo* antimicrobials based upon the minimal length of 24 residues for acheiving maximal antimicrobial potency. The observation that these lead compounds are broadly active against our prototype gram-positive and -negative organisms, including mucoid and non-mucoid clinical isolates of *P. aeruginosa*, suggests that these peptides may be a template on which to base further derivatives.

A *de novo* approach to peptide synthesis using natural amino acids is now predictable based on a comparison of the large number of host derived peptides (that exhibit a cationic amphipathic motif) described over the past two decades (69, 140). A review of the antimicrobial peptide literature raises the question of why a single sequence has not evolved as a predominant antimicrobial peptide functioning in the innate immunity of all vertebrate species. It seems that

this cationic amphipathic motif has evolved in its place. One possibility is that peptides with the same motif but with unique sequences behave differently against different bacterial pathogens in a variety of environments.

McLaughlin and co-workers initiated the concept of multimeric peptide design from a unique sequence (251). However, they limited their studies to peptides with a maximum length of 21 residues and thus did not allow for a complete appreciation of the effects of length on antimicrobial activity. In addition, they used Lys as their predominant cationic residue. In contrast, we exploited the membrane-active properties of Arg that have been documented in the literature (370, 417, 420). We utilized Val on the hydrophobic face because of its predominance in the LLP1 derivatives that we have described previously (395, 396). Finally, substitution of the membrane-seeking amino acid Trp (355, 417) in the hydrophobic faces in the WLBU series also allowed us to appreciate its influence on antimicrobial activity and selectivity. The possibility of using only three amino acids and synthesizing longer and more potent peptides in the form of a base unit is a novel aspect of these studies that affects the economic feasibility of peptide synthesis for large scale antibiotic production. For example, the anti-HIV1 drug T20 is synthesized through the condensation of peptide fragments (216, 247, 404).

We have shown that the activity of the LBU peptides was generally improved with increased chain length, with 24-residue peptides achieving optimal antibacterial selectivity. Although antibacterial activity correlated consistently with helicity for the LBU series, the length-potency correlation could be explained by better optimization of both the hydrophilic and hydrophobic faces with increasing length. However, hydrophobicity is the prime candidate in the observed increase in mammalian cytotoxicity. LBU2, the most amphipathic (mean hydrophobic moment of 0.80) (177) and highly helical in this series, has the highest Arg-to-Val ratio (13/11),

thereby favoring strong initial interactions between the peptide and the highly electronegative LPS. This is supported by the observation that LBU3, with 36 residues and a lower hydrophobic moment (0.70), showed no net gain in activity in comparison to LBU2 against *P. aeruginosa*.

The Gram-negative *P. aeruginosa* has a double membrane, whereas *S. aureus* only a cytoplasmic membrane. An important question is why WLBU2 displays similar activities against both *P. aeruginosa* and *S. aureus* or why LBU2 is more potent against *P. aeruginosa* than against *S. aureus*. While higher activity against the single-membrane bacterium would be the apparent prediction, a better explaination takes into consideration the types of interactions as the most important antimicrobial determinant. The surface of *P. aeruginosa* is more electronegative than that of *S. aureus*. Therefore, stronger electrostatic interactions between LBU2 and the former would energetically favor the insertion of LBU2 into the *P. aeruginosa* outer membrane. Once LBU2 penetrates the outer membrane, it is possible that the barrier to the cytoplasmic membrane of this bacterium (*e.g.*, thinner cell wall) is much weaker than that of the single membrane of *S. aureus*. The Trp inclusion in the LBU2 derivative, WLBU2, enhanced the interaction between the peptide and the cytoplasmic membrane of *S. aureus*, possibly by stronger hydrophobic (in addition to electrostatic) interactions. By the same analysis, the peptide affinity would be even weaker for the surface of eukaryotic cells, which is much less electronegative than that of Gram-positive bacteria. An important concern is the transducing potential of these peptides due to the high proportion of Arg (>50%) in the primary sequence of LBU2 or WLBU2. The penetration of the eukaryotic cell membrane by the peptide could result in the destruction of the *P. aeruginosa*-like double membrane of the mitochodria and apoptosis. However, the concentration threshold for cytoplasmic membrane lysis or penetration is obviously higher for

eukaryotic than bacterial cells, which is supported by the observation that WLBU2 is toxic to both bacterial organisms at concentrations that do not affect the viability of the host cells.

The peptide WLBU2, the most amphipathic of the WLBU series, was also the most potent in both PB and PBS and retained anti-pseudomonal selectivity presumably because of its high hydrophobic moment ($\mu = 0.83$) (177). Moreover, the three Trp residues in the hydrophobic face rendered WLBU2 considerably active against *S. aureus* without significantly affecting its mammalian cytotoxicity. We are currently investigating the antibacterial activity of WLBU2 in human serum and its immunomodulatory effects on primary epithelial cells. This information will be relevant to the potential *in vivo* efficacy of *de novo* engineered antimicrobial peptides.

2.6. ACKNOWLEDGEMENTS

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3. CHAPTER 3: ACTIVITY OF THE *DE NOVO* ENGINEERED ANTIMICROBIAL PEPTIDE WLBU2 AGAINST *PSEUDOMONAS AERUGINOSA* IN HUMAN SERUM AND WHOLE BLOOD: IMPLICATIONS FOR SYSTEMIC APPLICATIONS

3.1. Abstract

Cationic amphipathic peptides have been extensively investigated as a potential source of new antimicrobials that can complement current antibiotic regimens in the face of emerging drug-resistant bacteria. However, the suppression of antimicrobial activity under certain biologically relevant conditions (*e.g.*, serum and physiological salt concentrations) has hampered efforts to develop safe and effective antimicrobial peptides for clinical use. We have analyzed the activity and selectivity of the human peptide LL37 and the *de novo* engineered antimicrobial peptide WLBU2 in several biologically relevant conditions. The host-derived synthetic peptide LL37 displayed high activity against *Pseudomonas aeruginosa*, but demonstrated staphylococcal-specific sensitivity to NaCl concentrations varying from 50 to 300mM. Moreover, LL37 potency was variably suppressed in the presence of 1 to 6mM of Mg^{2+} and Ca^{2+} ions. In contrast, WLBU2 maintained its activity in NaCl and physiologic serum concentrations of Mg^{2+} and Ca^{2+}. WLBU2 was able to kill *P. aeruginosa* (10^6 cfu/ml) in human serum, with an MBC <9μM. Conversely, LL37 was inactive in the presence of human serum. Kinetic assays of bacterial killing in serum revealed that WLBU2 achieved complete bacterial killing by 20min. Consistent with these results was the ability of WLBU2 (15-20μM) to eradicate bacteria from *ex vivo* samples of whole blood. The selectivity of WLBU2 was further demonstrated by its ability to specifically eliminate *P. aeruginosa* in co-culture with human monocytes or skin fibroblasts without detectable adverse effects to the host cells. Finally, WLBU2 displayed potent efficacy against *P. aeruginosa* in an intraperitoneal infection model using female Swiss Webster mice. These results establish a potential application of WLBU2 in the treatment of bacterial sepsis.

3.2. Introduction

The emergence of multiple drug resistance among bacterial pathogens has stimulated the search for new anti-infective agents that can complement current antibiotic regimens (133, 214, 301, 312, 349, 368). Cationic amphipathic peptides (CAPs) have been extensively investigated as a potential source of these agents (212, 252, 312) and are a major class of antimicrobial peptides with different secondary structures (α-helix, β-sheets, loops, *etc.*) that provide neutrophils and epithelial surfaces of multicellular organisms with a rapid and efficient means of inactivating invading pathogens (27, 222, 224, 257, 340, 405, 410, 444). These ubiquitous peptides typically consist of 20 to 40 amino acid residues and are highly cationic. Most host-derived CAPs display broad activity against both gram positive and negative bacteria (27, 49, 52, 63, 107).

The mechanisms of action of antimicrobial peptides, although not completely elucidated, have been widely studied. The interactions of CAPs with their microbial targets are thought to occur electrostatically and can be mediated by lipid molecules on bacterial surfaces (27, 192, 239, 244). Numerous studies of CAP interactions with variably charged liposomes suggest that antimicrobial peptides selectively bind bacterial over eukaryotic cell membranes because of the negatively charged lipids found on the outer leaflets of the former (72, 153). The mechanisms of the antitumor and antiviral properties of CAPs are thought to be related to the presence of negatively charged phosphatidylserine molecules on the surfaces of tumor cells (118, 416) and sialic acid and heparan sulfate associated with viruses (25, 118, 148, 254). CAP antifungal (62, 121) and immunomodulatory properties (3, 50, 199, 281) have also been reported.

These peptides offer an appealing alternative as antimicrobial agents that can be used to combat multidrug resistance. Some bacteria display decreased susceptibility to several antimicrobial peptides by modifying their LPS or lipotheic acid molecules, leading to a decrease

in net negative charge on the bacterial surface (94, 109). However, unlike the selection of resistance observed with current antibiotic treatments (333), the emergence of CAP-resistant phenotypes via multiple passages of bacterial strains is uncommon. In comparison to current antibacterial drugs, CAPs have the ability to kill bacteria very quickly (within 30 to 180sec) (396). Rapid killing allows little time for a particular genetic mutation in a bacterial cell to result in successful changes in the lipid bilayer, a process that likely requires modification of enzymatic pathways in addition to nucleotide or protein targets.

Nevertheless, there are multiple obstacles to developing peptides of therapeutic potential. Despite the broad activity of host-derived peptides, their potency is often optimal only under specific conditions. For instance, several CAPs, although highly active in phosphate buffer, display a significant decrease in antibacterial potency in the presence of physiological concentrations of NaCl and divalent cations (93, 351, 391, 403, 437). Please note that the increased resistance to physiological NaCl concentration due to the inclusion of Trp residues in the LBU derivatives was discussed in the Chapter 2. In some cases (*e. g*., cystic fibrosis airway fluids), hypo-osmolar NaCl may be sufficient to significantly suppress antimicrobial function (209). Thus, the development of CAPs has been directed toward overcoming some of these challenges by either modifying host-derived peptides (188) or selecting specific amino acids for *de novo* design (252, 327, 330). Although several newly engineered CAPs demonstrate a significant degree of salt resistance (162, 163, 188, 330), current studies have not indicated that these peptides can overcome the obstacles encountered in physiological fluids and systems. Such limitations have shifted the focus upon developing CAPs as topical agents (152).

We previously reported the *de novo* design of modular CAPs comprised of multimers of 12-residue lytic base unit (LBU) sequences of Val and Arg, with Trp substitutions (the LBU and

WLBU series, respectively). In these peptides, the hydrophilic (Arg) and hydrophobic (Val and Trp) domains were maximally segregated (by 180°) and optimized as idealized amphipathic helical structures. The characterization of the activities of the LBU and WLBU peptide series against *P. aeruginosa* and *Staphylococcus aureus* led to the selection of WLBU2 (a 24-mer) as the shortest peptide with the highest antimicrobial potential, high helical propensity, and little toxicity to mammalian cells (162).

The engineered peptide derivative WLBU2 (RRWVRRVRRWVRRVVRVVRRWVRR) is composed predominantly of Arg (13 residues) and Val (eight residues), with three Trp residues in the hydrophobic face separated from each other by at least seven amino acids. The residues in WLBU2 were arranged to form an idealized helical and amphipathic structure, with optimal charge and hydrophobic densities (162). By comparison, LL37 (LLGDFFRKSKEKIGKEF KRIVQRIKDFLRNLVPRTES) is an α-helical peptide with mainly Lys and Arg in the hydrophilic face and several different hydrophobic amino acid residues (Phe, Val, Ile, *etc.*) in the hydrophobic domain. LL37 has distinct cationic and hydrophobic domains with an amphipathic structure that is not as optimized as that of WLBU2.

Based on these subtle but important structural differences, we sought to compare the potency of WLBU2 to that of LL37 in rigorous test conditions to demonstrate how certain limitations of host antimicrobial agents can be overcome using newly designed peptides with optimized amphipathic structures. To conclude these studies, the *in vivo* efficacy of WLBU2 was examined in a mouse model of *P. aeruginosa* bacteremia. The results of our investigations suggest that WLBU2, in contrast to LL37, is an antimicrobial agent that may have applications to systemic infections.

3.3. Materials and Methods

3.3.1. Peptide Synthesis

The peptides WLBU2 and LL37 were synthesized using standard FMOC synthesis protocols as previously described (396). Synthetic peptides were characterized and purified by reverse-phase HPLC on Vydac C18 or C4 columns (The Separations Group, Hesperia, CA), and the identity of each established by mass spectrometry (Electrospray Quatro II triple quadruple mass spectrometer, Micromass Inc., Manchester, UK). Peptide concentrations were determined using a quantitative ninhydrin assay as previously described (396). A peptide sample of known concentration was used to evaluate several stocks of WLBU2 by spectrophotometric analysis, based on Trp absorbance at 280nm. By plotting different absorbances at 280 nm against peptide concentrations, a standard curve was generated from which concentrations of WLBU2 were deduced.

3.3.2. Bacterial Killing Assays

Bacterial killing assays were conducted as previously described (162, 396) using the *P. aeruginosa* strain PA01 (amp^r) provided by Dr. Barbara Iglewski (University of Rochester, NY). In selected studies, a methicillin resistant strain of *S. aureus* (MRSA) (obtained from Children's Hospital of Pittsburgh Microbiology Laboratory) was used as a target bacterial strain. To test the susceptibility of these index bacteria to the peptides described, the standard NCCLS microbroth dilution assay had to be modified to compare the influence of NaCl, Mg^{2+}, Ca^{2+}, sera, or whole blood on the activity of WLBU2 and LL37. The medium used was 10mM phosphate buffer, pH 7.2, (PB) to which the aforementioned substances were added as described in each experiment. In all except the kinetic experiment, exposure time was held at 30 min. Bacterial suspensions

(*ca.* 1×10^6 cfu/mL) in PB or containing NaCl, Mg^{2+}, Ca^{2+}, sera, or whole blood as described were incubated with two-fold dilutions of peptides for 30min at 37°C. Following treatment, the bacterial samples were plated on tryptic soy agar (TSA) (Difco, Detroit, MI) to assay viable bacteria. Surviving colonies were counted the following day to determine the minimum bactericidal concentration (MBC), defined as the molar concentration of peptide reducing the viable bacteria within a suspension by three orders of magnitude. Results were expressed on a molar basis as an average of MBC values obtained from two to five independent experiments. This assay was also performed in the presence of heat-inactivated human serum (306) or plasma, ACES buffer (0.05mM ACES, 0.12mM NaCl, pH 7.34), or ACES-based $CaCl_2$ or $MgCl_2$ (Sigma, St Louis, MO), as indicated. As a substitute for PB, ACES buffer was used to avoid the precipitation of calcium or magnesium phosphates.

3.3.3. Kinetics of Bacterial Killing

The procedure for bacterial killing assays was modified to determine the rate of bacterial killing by WLBU2 and LL37. *P. aeruginosa* strain PA01 (*ca.* 1×10^6 cfu/mL) was treated with 15μM peptide in human serum (isolated from blood samples taken from healthy donors) and 1μM in the presence of PBS. Aliquots of 20μL of the peptide-treated suspension were withdrawn at different times from 0 to 30 min at room temperature, serially diluted, and plated on TSA to determine bacterial counts. Average values for triplicates were expressed as log cfu/mL plotted against time (min).

3.3.4. Selective Toxicity in Co-culture Systems

3.3.4.1. *Selective Toxicity in Human Whole Blood*

Heparinized human blood from healthy donors (with the approval of the University of Pittsburgh Institutional Review Board) was inoculated with mid-log phase *P. aeruginosa* PA01 cells (ca. 1×10^6 cfu/mL) and then treated with varying concentrations of peptide ranging from 0-50μM to a total volume of 550μL. The reaction mixture was incubated at 37°C for 60min with gentle shaking. To determine viable bacterial count, each sample was serially diluted using 20μL as described previously. For parallel analysis of red blood cell (RBC) lysis, the remaining suspension was spun at 14000rpm (20,000 g) in an Eppendorf centrifuge model 5417C (Brinkmann Instruments, Westbury, NY) for 4min, and 50μL of the supernatant (hemoglobin fraction) diluted in 450μL distilled water. Similarly, 0-50μL untreated blood was diluted with water to a final volume of 500μL, and the supernatant (hemoglobin suspension) used to produce a standard curve of RBC lysis. The average absorbance values of the supernatants of all samples (200μL) were measured in duplicate in a plate reader at 570nm as a measure of hemoglobin released from lysed cells. Red blood cell lysis buffer (8.3g/L ammonium chloride in 0.01M Tris-HCl, pH 7.5, Sigma, St. Louis, MO) was used as a control (50μL whole blood and 450μL buffer). Percent RBC lysis and bacterial survival were compared to determine the selective potential of the peptides. These experiments were verified by four independent trials.

3.3.4.2. *Co-culture of Human Cells with* P. Aeruginosa

To determine the selectivity of WLBU2 for bacteria versus human cells, the activities of WLBU2 and LL37 were assayed in a co-culture of human monocytes or primary human skin

fibroblasts (HSF) with *P. aeruginosa*. Peripheral blood monocytes were isolated from buffy coats obtained from sterile and heparinized human blood samples. Using histopaque gradient centrifugation (Sigma, St. Louis, MO), PBMC were isolated, and monocytes subsequently removed by adherence on 2% gelatin (Sigma, St. Louis, MO) plates. After isolation, the monocytes in RPMI were transferred to a 96-well plate (10^5 cells/well in RPMI or IMDM) and incubated at 37°C and 6% CO_2 until a monolayer was formed. The medium was aspirated, and a 100µL suspension of *P. aeruginosa* PA01 (2×10^6 cells/mL in 99% human serum) was added to each well. The bacterial suspensions over the eukaryotic cell monolayer were further diluted to10^6 cells/mL with equal volumes of peptide at various concentrations (0 to 100µM) in 99% human serum. The co-culture was then incubated at 37ºC for 30min. To determine bacterial survival, the co-culture medium was serially diluted (after gentle pipetting) up to 1:1000 by transferring 20µL aliquots to another 96-well plate containing 180µL PBS per well. Each dilution was plated (100µL) on tryptic soy agar (TSA) and incubated overnight at 37°C. Bacterial counts were then determined and expressed as a logarithm of colony forming units per mL (log cfu/mL).

To further characterize the selectivity of WLBU2 with regard to *P. aeruginosa* and human cells, this co-culture assay was also performed using human skin fibroblasts (HSF) (passage 20) in Iscove's Modified Dulbecco's Medium (IMDM) (Invitrogen, Grand Island, NY)

Measurement of host cell viability post-peptide treatment was accomplished using a tetrazolium-based colorimetric assay (237, 457). After two consecutive gentle washes with PBS, the cells were incubated in 100µL IMDM (HSF) or RPMI (monocytes) containing 10% FBS (vol/vol) and 0.5mg/mL MTT Formazan (MTT) (Sigma, Lakehood, NJ). The reaction mixtures were incubated at 37^{o}C in 5% CO_2 for 4-6h after which equal volumes of 0.1N HCl:isopropanol

were added to dissolve the resulting blue crystals. The percent viability was assessed by taking absorbance measurements at 570nm using the Dynatech MR5000 96-well plate reader (Germantown, MD). For controls, cells were first treated with 100% serum (the test medium) in the presence or absence of bacteria, or with 100% lysis buffer [8.76g NaCl, 10g DOC, 1M Tris HCl (pH 8.0), and 10g Triton X 100, per liter of dH_20] in the absence of bacteria. The experiments were performed in triplicate, and viability data averaged. The final toxicity values were expressed as mean percent toxicity for each test condition minus any observed toxicity to the human cells by bacterial treatment alone.

3.3.5. Host Toxicity and Proliferation Assays in the Absence of Bacteria

To investigate the influence of WLBU2 on human cells in the absence of bacteria, we sought to determine whether long-term peptide treatment of human cells would adversely affect cell viability or functionality. Thus, freshly isolated human blood monocytes were treated with 15μM WLBU2 or LL37 for 48h at 37°C and 6% CO_2 in human serum. Cell viability in the presence or absence of peptide was evaluated by MTT staining as described previously. To characterize the influence of the peptides on lymphocytic proliferation, peripheral blood mononuclear cells (PBMC) were isolated and treated with 15μM peptide in RPMI-based 70% fetal bovine serum for 30min, at 37°C and 6% CO_2. Subsequently, equal volumes of the mitogens, PMA (50ng/mL) and ionomycin (250ng/mL) in RPMI were added to each well. After 3 days, ^{3}H-thymidine (1μCi/well in 50μL RPMI) was added to the test and control (no peptide and/or unstimulated) wells. Following incubation for 16-18h, the cells were harvested onto a filtermat using a Tomtec cell harvester (Hamden, CT), and incorporation of ^{3}H-thymidine detected in a Wallac Microbeta Liquid Scintillation Counter (Turku, Finland). To calculate

stimulation indices, radioactive counts per min (cpm) for mitogen-treated (stimulated) samples were divided by cpm for untreated samples (unstimulated).

In vivo *Toxicity*

Before evaluating the antibacterial efficacy of WLBU2 *in vivo*, it was essential to examine its toxic potential, which was performed via intravenous (IV) administration. Female Swiss Webster mice (Taconic, Germantown, NY) were injected IV with 0.1mL PBS and 3, 6, 12, or 16mg WLBU2 per kg body weight. Each dose was administered twice within 24h, and all mice (10/group) were monitored for signs of toxicity such as weight loss, piloerection, motility, histopathology, and survival.

3.3.6. *In Vivo* Toxicity

Before evaluating the antibacterial efficacy of WLBU2 *in vivo*, it was essential to examine its toxic potential, which was performed via intravenous (IV) administration. Female Swiss Webster mice (Taconic, Germantown, NY) were injected IV with 0.1mL PBS and 3, 6, 12, or 16mg WLBU2 per kg body weight. Each dose was administered twice within 24h, and all mice (10/group) were monitored for signs of toxicity such as weight loss, piloerection, motility, histopathology, and survival.

3.3.7. Intraperitoneal Bacterial Inoculation Followed by Intravenous Antibacterial Therapy

Mid-log phase bacteria *(P. aeruginosa* PA01 amp^r), were prepared in sterile PBS to achieve the desired bacterial suspensions for intraperitoneal (IP) injections. Prior to examining the efficacy of WLBU2 *in vivo*, the minimum *Pseudomonas* lethal dose (PLD), the minimum

dose causing 100% mortality, was determined (1×10^7 cfu) and used in a 0.5 ml volume to inject bacteria IP. The animals were then randomized to receive IV isotonic sodium chloride solution (control group), or 3 mg/kg WLBU2 30 to 45min after bacterial challenge. The animals in each group, which included 14 mice (7/group for each of 2 independent trials), were returned to individual cages and subsequently monitored for 7-10d. The end points of the study were indicated either by 7-10d survival or by complete absence of motility and presence of hypothermia (Thermistor thermometer, Kent Scientific, Torrington, Connecticut) as signs of terminal illness or lethality.

3.3.8. Evaluation of Treatment

Quantitative blood cultures on carbenicillin TSA plates (200µg/ml) were performed to determine bacterial loads over the course of the infection. Blood samples were obtained from the tail vein by aseptic percutaneous puncture 0.5h to 36h after bacterial challenge and serially diluted. Then, a 0.1mL volume of each dilution was spread on carbenicillin TSA plates and incubated at 37°C overnight for enumeration of developed colonies. At the disease endpoint, animals were euthanized, and tissues weighed and homogenized using 70µm cell strainers (Becton Dickinson, Franklin Lakes, NJ) to determine bacterial cfu/g tissue. To compare fatality rates between different groups (treated and mock-treated), the Log-rank test was performed using GraphPad Prism version 3.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com. Significance was accepted when P values were less than 0.05.

Table 4. Influence of sodium, magnesium, and calcium chloride on antibacterial activity [a]

Salt (mM)		MBC (μM)	
		LL37	WLBU2
NaCl	0	0.5	0.5
	50	0.5	0.5
	150	0.5	0.25
	300	1.2	0.5
$MgCl_2$	0	0.5	<0.5
	1	<2.5	<0.5
	3	>5	<0.5
	6	>5	<1
$CaCl_2$	0	0.5	0.5
	1	>5	0.5
	3	>5	<1
	6	>5	<2

[a]*P. aeruginosa* PAO1 was incubated with 2-fold serially diluted peptides at 37°C for 30 min. Bacterial survival at corresponding peptide concentrations was evaluated by broth dilution assays as described in Materials and Methods. Sodium chloride, even above physiological concentration, had negligible effects on the activities of LL37 and WLBU2 against P. aeruginosa. However, LL37 showed an Mg^{2+}- and Ca^{2+}-dependent decrease in potency, while the activity of WLBU2 was slightly inhibited at 6mM divalent cations. The MBCs were derived from representative values of two-three independent experimental trials. MBC = minimal bactericidal concentration.

3.4. RESULTS

3.4.1. Influence of Physiological Salt Concentrations on Antipseudomonal Activity

As previously mentioned, the activity of host-derived antimicrobial peptides is often inhibited in the presence of physiological serum concentrations of sodium chloride and divalent cations (304, 403). For instance, we initially demonstrated that LL37 was inactive against *S. aureus* in PBS (162). However, the activity of the *de novo* engineered peptide WLBU2 was not altered under such conditions. To further examine the influence of salt on antibacterial activity, we treated *P. aeruginosa* with different peptide concentrations in varying NaCl conditions up to 300mM. As indicated in Table 4, the peptides were highly toxic to *P. aeruginosa* in NaCl (at all concentrations), with MBC ranging from 0.5-1 μM (2.3-4.5μg/mL) for LL37 and ≤0.5μM (1.7μg/mL) for WLBU2. However, LL37 displayed a significant salt-dependent decrease in activity against MRSA, with an MBC greater than 2μM in as little as 50mM NaCl (data not shown). In contrast, the activity of WLBU2 against MRSA remained unchanged under varying NaCl conditions, with MBCs <0.5μM (data not shown). These results provide evidence that, unlike LL37, WLBU2 has the ability to resist broad changes in NaCl concentrations regardless of the test organisms (gram-negative *P. aeruginosa* or gram-positive MRSA).

Divalent cations bridge lipopolysaccharides on bacterial surfaces, suggesting that they may serve as competitive inhibitors to CAPs. To investigate whether divalent cations can inhibit peptide activity, we determined the dose-dependent effects of Mg^{2+} and Ca^{2+} on peptide activity using 1, 3, and 6mM $MgCl_2$ and $CaCl_2$ in ACES buffer (normal physiological serum concentrations of Mg^{2+} and Ca^{2+} are in the range of 2 ± 0.5mM). The activity of LL37 was significantly inhibited in a dose-dependent manner, as MBCs were greater than 5μM at 3mM Mg^{2+} (Table 4). In contrast, WLBU2 activity was unaffected at concentrations as high as 3mM

Mg^{2+} (MBC <0.5). However, there was a small increase in MBC (<1μM) at 6mM. The effects of Ca^{2+} on the activity of both peptides were slightly more apparent, with LL37 not reaching an

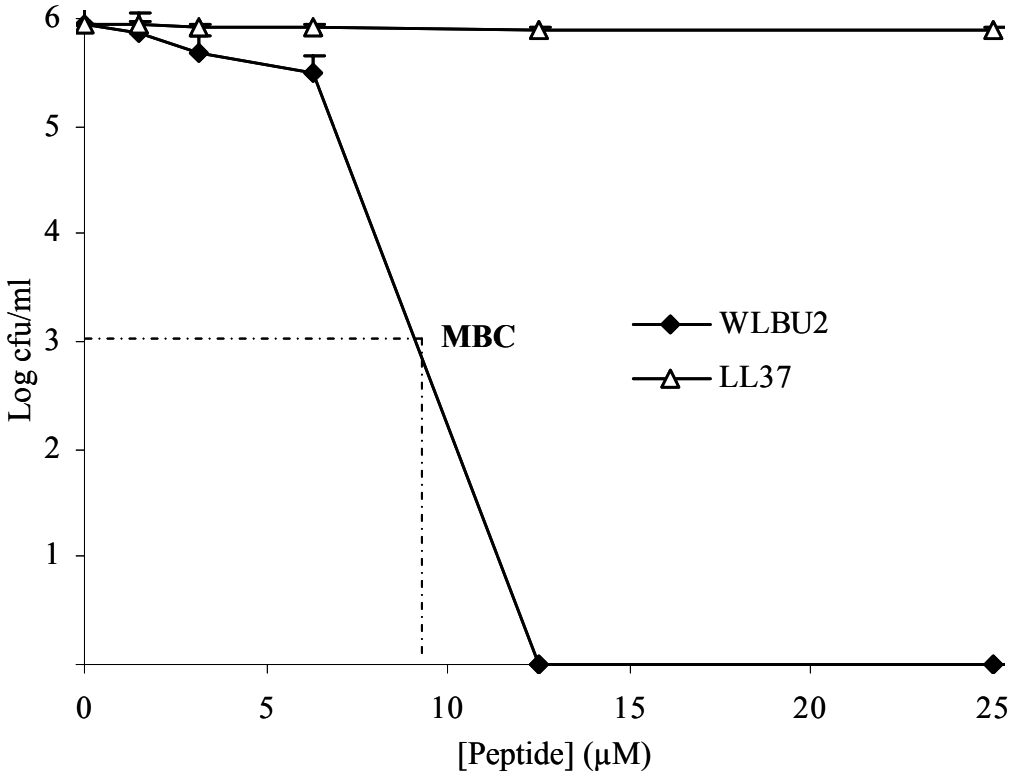

Figure 7. Activity of WLBU2 in human serum

To determine activity in human plasma, bacterial isolates of *P. aeruginosa* PAO1 (~10^6cells/ml) were incubated with 2 fold serially diluted peptides at 37°C for 30 min either in heat-inactivated human plasma or serum. Bacterial survival at corresponding peptide concentrations was evaluated by broth dilution assays as previously described. WLBU2 was able to sterilize the bacterial suspension at 12.5 μM, while LL37 demonstrated no significant activity even at 100 μM (not shown). Results of activity in serum and plasma were identical. The graphs were derived from average values of four independently generated and almost identical triplicate sets of data. MBC = minimal bactericidal concentration.

MBC within 0-5μM even in as low as 1mM $CaCl_2$. WLBU2 was four-fold less active in calcium concentrations varying from 1mM (MBC = 0.5μM), to 6mM (MBC <2μM) (Table 4). Notably, calcium ions only had a minor inhibitory effect on WLBU2 (MBC <1μM) within physiological

concentration range (1.5-2mM). These results suggest that WLBU2 has been successfully engineered to be unaffected by broad variations in NaCl and divalent cation concentrations found to be challenging for host-derived peptides.

3.4.2. Antibacterial Activity and Selectivity in Human Serum

The inactivation of CAPs in human serum has hindered efforts to develop antimicrobial peptides for systemic use (152, 246). As a consequence, less challenging conditions (*e.g.*, phosphate buffer) are commonly used to examine antibacterial activity and host toxicity. Moreover, the characterization of antimicrobial selectivity has been based on the exposure of mammalian cells to peptides in the absence of microbial pathogens in media or buffer (*e.g.*, red blood cell lysis assay in PBS) (396). To evaluate antibacterial selectivity more appropriately, we have developed a comprehensive series of experiments in which both bacteria and host cells are mixed prior to the addition of peptide. This assay was previously described in a study in which HSF and *P. aeruginosa* PA1244 were combined and then treated with peptide in PBS-based IMDM (162). Although this assay provides important information about the selective target of a peptide, it remains unclear whether similar results would be expected under biological conditions.

To investigate the potential of WLBU2 for systemic applications, we initially examined the influence of human serum on its antibacterial activity and compared it with that of LL37. Thus, *P. aeruginosa* suspensions were treated with increasing peptide concentrations in the presence of human serum (98%). As indicated in Figure 7, LL37 displayed no significant activity even at concentrations up to 100μM (data not shown). By contrast, WLBU2 was highly active against *P. aeruginosa*, with no bacterial survival observed at 12.5μM (MBC of <9μM). Because peptide clearance may have a profound impact on activity *in vivo*, we compared the rates of

bacterial killing in human serum and in PBS (Figure 8). The results of this experiment indicate that the peptide WLBU2 requires at most 20min to kill about 1×10^6 cfu/ml of *P. aeruginosa* at

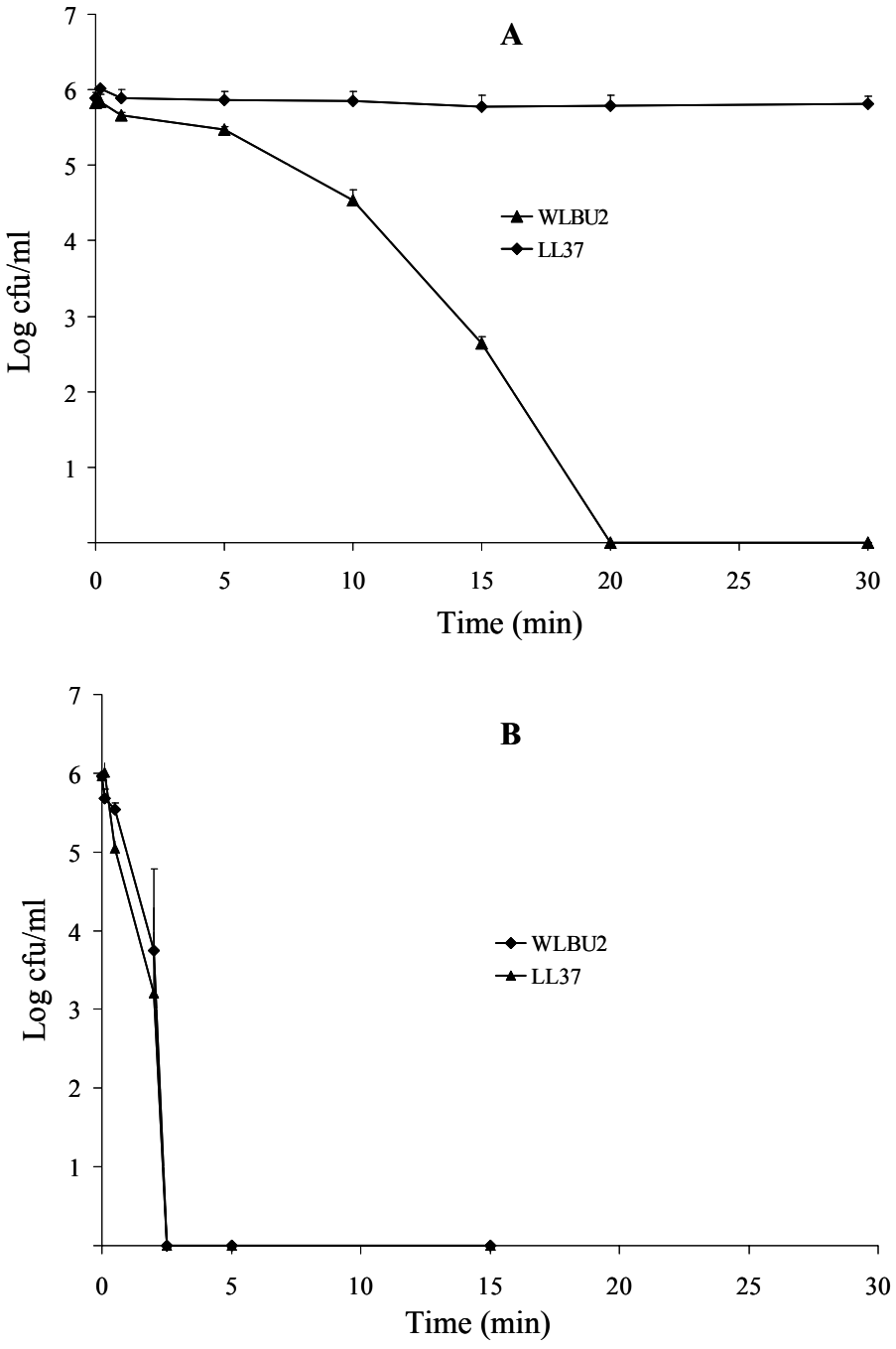

Figure 8. Kinetics of bacterial killing

P. aeruginosa PAO1 was treated with 15 μM peptide in human serum (A) and 1μM in PBS (B). Bacterial survival was monitored over time (up to 30 min) and determined as described in Materials and Methods. The results reveal that a minimum period of 20 min is required for complete bacterial killing in about 98% human serum. The peptide LL37 was inactive under this condition as expected. However, both LL37 and WLBU2 achieved complete killing within the first 5 min of treatment in PBS. Data plotted are representative average values for one triplicate set from 3 independent experiments.

15μM in approximately 98% human serum. As expected, LL37 did not show significant activity over time in human serum. Not surprisingly, both peptides sterilized a similar bacterial suspension of *P. aeruginosa* PA01 (1×10^6 cfu/ml) in PBS at a concentration of 1μM in less than 5min. These data suggest that WLBU2 should be further investigated for systemic use.

The higher MBC of WLBU2 in human serum raised a concern for potential toxic effects on mammalian cells. Moreover, it remained unclear whether the complete cellular composition of whole blood would affect antibacterial potency. To address these two issues, we examined the antibacterial selectivity of WLBU2 in human whole blood. In this system, a bacteremic condition was established *ex vivo* with *P. aeruginosa* PA01 (1×10^6 cfu/ml) prior to addition of peptide. Bacterial survival was determined by serial dilution assay, and red blood cell lysis was deduced from a standard curve generated by spectrophotometric analysis of water-treated blood. As predicted, LL37 did not show any antibacterial activity against *P. aeruginosa* (data not shown). Conversely, WLBU2 demonstrated the ability to "sterilize" the *Pseudomonas*-inoculated blood at 15-20μM peptide (Figure 9), consistent with its activity in human serum. In addition, there was no detectable toxicity to the erythrocytes at all test concentrations (up to 50μM). These results further underscore the enhanced potency of WLBU2 over the host peptide LL37 and may serve as a preliminary indicator of the antibacterial potential of WLBU2 *in vivo*.

Because the blood cell lysis assay did not reveal any specific information about the fate of the leukocytes in the *ex vivo* bacteremic model, the cytotoxic effect of the peptide on human

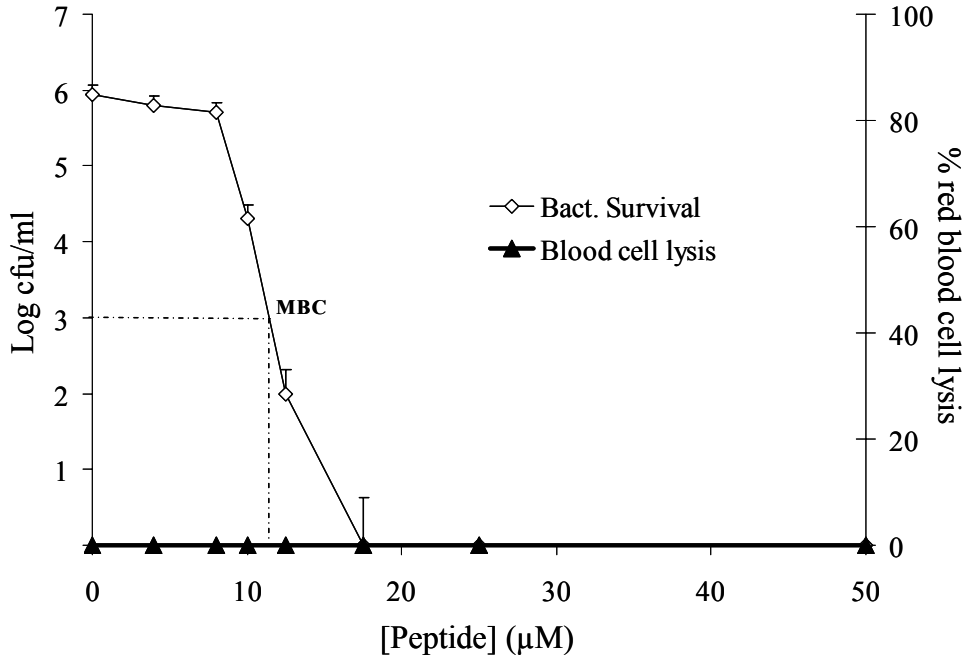

Figure 9. Peptide efficacy in an *ex vivo* bacteremic model

Human whole blood was inoculated with *P. aeruginosa* PAO1 (~10^6cells/ml) and treated with peptide at various concentrations for 45 min at 37°C. Bacterial count was determined by standard bacterial dilution assay and blood cell lysis by spectrophotometric analysis of hemoglobin release. The erythrolytic effect of red blood cell lysis buffer was comparable to that of water, which was used to generate the standard curve (not shown) in this assay, as described in materials and Methods. The peptide WLBU2 was remarkably selective against the bacterial cells, with no detectable lytic effects on the blood cells. The graph was derived from average values of four independently generated and almost identical triplicate sets of data. MBC = minimal bactericidal concentration.

blood monocytes was investigated. To accomplish this, cultures of freshly isolated peripheral blood monocytes were inoculated with *P. aeruginosa* in heat-inactivated human plasma or serum (306). Subsequently, the co-culture was treated with peptides as described in Figure 4. Using a tetrazolium-based staining method (MTT), the level of toxicity to the monocytes was determined. WLBU2 did not affect the viability of the monocytes (0% cytotoxicity).

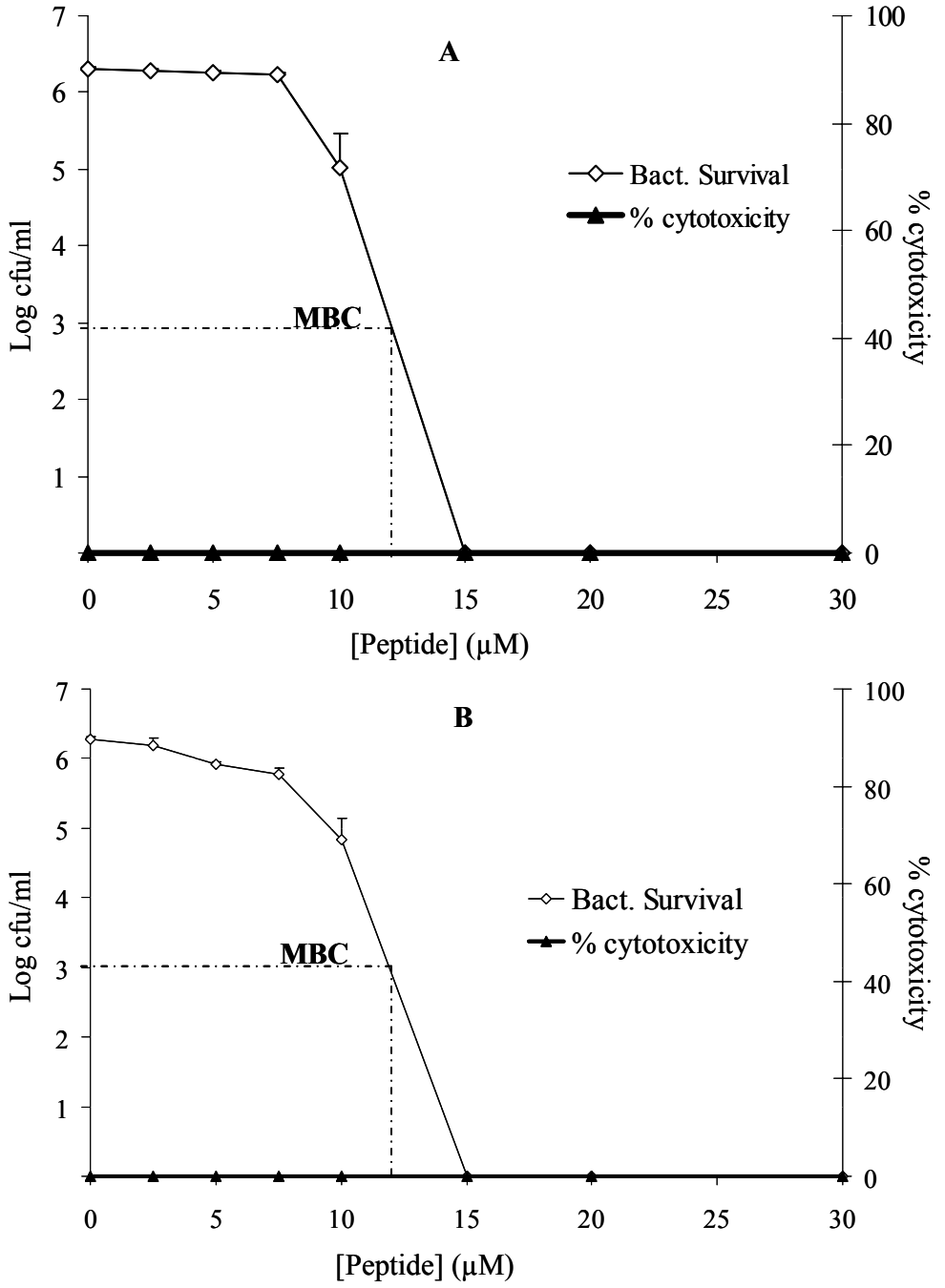

Figure 10. WLBU2 selectively targeted *P. aeruginosa* in a co-culture model in human plasma

Approximately 10^5 Human monocytes (A) or primary human skin fibroblasts (HSFCCD-986sk) at passage 20 (B) were inoculated with *P. aeruginosa* in 98% heat-inactivated human serum. The co-culture was then incubated for 60 min with 2-fold dilutions of peptides as described in Materials and Methods. Bacterial survival was determined as in standard broth dilution assay and

the percent viability of the human PBMC or HSF evaluated by MTT staining. The peptide WLBU2 displayed high antibacterial selectivity in comparison to the host-derived peptide LL37 (not shown). Data plotted are representative averages from 3 independent experimental trials. MBC = minimal bactericidal concentration.

Consistent with antibacterial activity in human blood, bacterial killing was optimal at 15μM peptide for both peripheral blood monocytes (Figure 4A) and primary HSF (Figure 4B). Again, LL37 was inactive under these conditions (data not shown). Taken together, these results underscore the high antibacterial selectivity of WLBU2 and identify it as a potentially effective and safe antimicrobial agent.

3.4.3. Effects of WLBU2 on Host Cells in the Absence of Bacteria

The results of the previous experiments demonstrate the effects of WLBU2 on human cells after only a short-term treatment. Furthermore, the influence of WLBU2 on the functionality of these cells remains unclear. To address these issues, the influence of WLBU2 on the viability of human PBMCs after exposure for 48h in human serum using MTT staining was determined. PBMC remained 100% viable in MTT assay (data not shown). Despite the evidence shown here in support for the safety of WLBU2 in a host environment, it could still be argued that, although nontoxic to mammalian cells in serum, WLBU2 could affect their functionality. To address this issue, we attempted to examine the influence of WLBU2 on lymphocytic proliferation. Thus, human blood lymphocytes were treated with 15μM peptide for 30min prior to the addition of PMA and ionomycin to determine the proliferation level by H^3-thymidine incorporation within 4d of stimulation (data not shown). The results show that lymphocyte proliferation either remained unchanged or increased by up to 15% for both LL37- and WLBU2-treated samples compared with untreated samples. On the whole, this study demonstrates that it

is possible to design antimicrobial peptides with optimized amphipathic helical structures for overcoming the challenges of physiological salt concentrations and biological fluids.

3.4.4. In Vivo Toxicity and Antipseudomonal Efficacy in an IP Infection Model

Both *in vitro* and *ex vivo* observations suggest the possible application of WLBU2 in the treatment of bacteremia. Thus, it was deemed logical to examine the potential therapeutic application of WLBU2 in a septicemic animal model of *P. aeruginosa* infection. Initially, we characterized the lethal potential of the peptide within a range of concentrations to determine the maximum tolerated dose (MTD). For these experiments, groups of 10 mice (25g each) were given two equal doses of WLBU2 IV varying from 3 to 16mg/kg body weight within 24h (Figure 5A). The peptide displayed no lethality or apparent toxic effect at up to 12mg per kg body weight in comparison with PBS-injected mice (not shown), but the mice receiving the highest peptide dose (16mg/kg mouse) died within 30min post-injection. no lethality, however, was observed in the other groups. Thus, the MTD for IV administration, the highest IV dose of WLBU2 that led to no obvious toxicity to the mice, was 12mg/kg. We are currently in the process of identifying the physiological basis for this toxicity.

Based on the toxicity profile of WLBU2, a bacteremic mouse model was developed to test the therapeutic potential of the peptide using female Swiss Webster mice. Initially, the minimum *P. aeruginosa* lethal dose (PLD) was evaluated by IP injections. It was determined that mice (~25g each) subjected to IP administration of 10^7 cfu (PLD) became terminally ill and required euthanasia within 36h (data not shown). IP-injected mice (10^7 cfu *P. aeruginosa*) treated IV with isotonic NaCl (PBS) became bacteremic after 2h post-injection (approximately 1000 cfu/mL blood). Bacterial loads increased to 1-10 billion cfu/mL of blood at the disease

endpoint (data not shown). Consistent with this finding was the observed dissemination

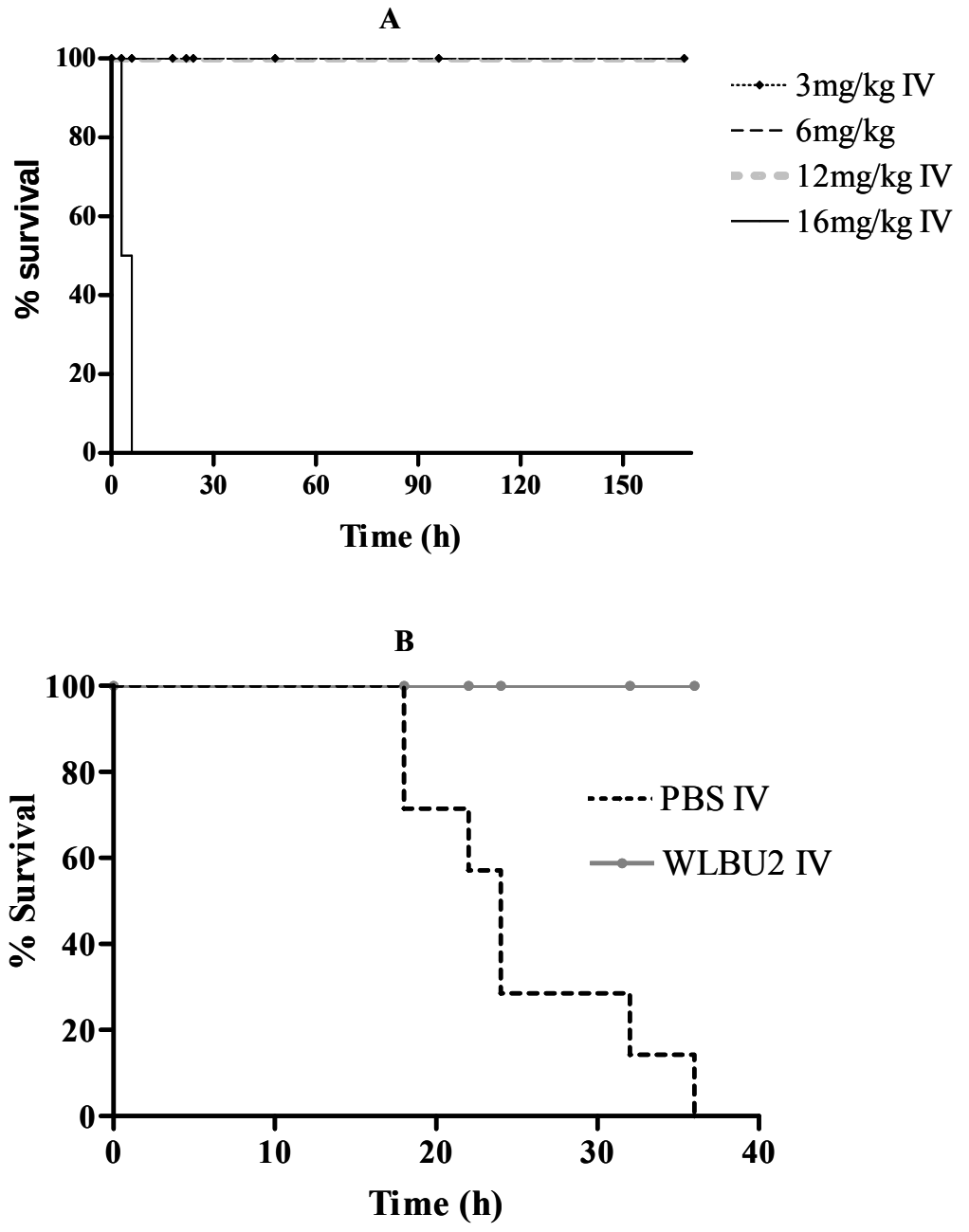

Figure 11. WLBU2 *in vivo* efficacy against *P. aeruginosa*

To characterize the *in vivo* efficacy of WLBU2, we first determined the maximum tolerated dose (MTD) IV by injecting female Swiss Webster mice with PBS or 3 -16mg WLBU2 per kg body weight IV. The animals were then followed for a minimum of 7 days for survival. Mice treated IV with 16mg WLBU2 per kg body weight died within 30min post-treatment. As shown in the Kaplan-Meier survival curve, the IV MTD is 12mg WLBU2 per kg body weight (A). Therefore,

the LD_{50} is between 12 and 16mg/kg. (B) The therapeutic potential of WLBU2 was determined by injecting mice IP with PAO1 (1.0-1.5 x 10^7 cfu). The animals were treated 30-45min post-injection with PBS, and bacterial load determined over time by blood culture on carbenicillin (200µg/mL) tryptic soy agar plates. The establishment of bacteremia began in about 2-3h (data not shown) and progressed to septicemia and fatality within 36h. In contrast, when *Pseudomonas*-infected mice were treated IV with 3 mg of WLBU2 per kg body weight 30-45min after the administration of the minimum PLD, not a single case of bacteremia and fatality was observed. A log rank test reveals a P value of less than 0.0001.

of bacteria to the internal organs (liver, lungs, kidney, and spleen), with bacterial loads varying from 10^8-10^9 cfu per gram of tissue during terminal illness (absence of motility and hypothermia). Identically inoculated mice injected IV with 3mg WLBU2 per kg mouse 30-45min after bacterial injections showed no signs of disease (normal motility, no piloerection, etc.). Moreover, no bacteria were recovered from the blood or the internal organs (liver, kidney, lungs and spleen) after 7-10d post-treatment (data not shown). Figure 5B illustrates a Kaplan-Meier survival analysis of the difference between mice treated IV with PBS and mice treated IV with WLBU2 30-45min after IP injection of bacteria. No lethality was observed in WLBU2-treated in comparison to mock-treated mice. It is concluded from this model that WLBU2 demonstrated high efficacy against *P. aeruginosa* PA01 *in vivo* when systemically administered.

3.5. DISCUSSION

In this study, the activity of a reference natural antimicrobial peptide (LL37) is compared with that of a *de novo* peptide (WLBU2) under biologically relevant conditions proven to be challenging to many of the most commonly studied antimicrobial peptides (152, 209). As shown in this study, LL37 displayed no saline sensitivity against *P. aeruginosa*, but its activity was variably suppressed in the presence of Mg^{2+} and Ca^{2+} and human serum. In contrast, WLBU2 was resistant to physiological concentrations of NaCl, $MgCl_2$, and $CaCl_2$. An important observation was the complete killing of *P. aeruginosa* by WLBU2 in human serum and whole

blood, without detectable adverse effects to human leukocytes, erythrocytes, and primary skin fibroblasts at concentrations five-fold above the MBC. However, the most critical finding was the antipseudomonal efficacy of WLBU2 in IP-infected mice.

Studies of natural peptides indicate that NaCl and divalent cations (even at subphysiologic levels) may affect CAP activity (209). In this study, LL37 sensitivity to NaCl for MRSA indicates that salt resistance is sometimes dependent on the test organism. Conversely, the highly cationic peptide WLBU2, a derivative of the *de novo* 24-mer sequence (LBU2), was shown to be highly potent against both *P. aeruginosa* and MRSA in similar NaCl concentrations. Based on the fact that this staphylococcal-specific salt sensitivity (displayed by LL37) was previously observed for the parent peptide LBU2 (162), it is evident that the strategic substitution of 3 Trp residues in the peptide hydrophobic face is likely to be responsible for the observed salt resistance against *S. aureus*. In support for this observation, several studies indicate that Trp may be an important determinant of antimicrobial activity (227, 228). For instance, a single substitution of Phe for Trp in lactoferricin considerably decreases its antimicrobial potency (416). In an attempt to examine the underlying mechanism of salt resistance, Park *et. al* demonstrated that salt resistance can be enhanced by increasing helical stability (327). As helical conformation is normally induced by peptide interaction with the bacterial surface, increasing the affinity of a peptide for the lipid bilayer should enhance its propensity to form a α-helix. In a saline environment, the hydrophobic and bulky rings of the Trp residues (by virtue of its membrane seeking property) are likely to enhance the affinity of WLBU2 for the bacterial membrane, thereby leading to a greater stability of the helical structure. However, raising the concentrations of Mg^{2+} and Ca^{2+} beyond serum physiologic levels had a minor inhibitory effect on the activity of WLBU2. This finding suggests that there is an optimal salt concentration (in

this case, twice the physiologic concentrations) beyond which the helical stability may be affected. One explanation could be that the divalent cations (at high concentrations) may more effectively compete with the peptide for the negatively charged bacterial surface. Nevertheless, an important lesson is that CAPs can be designed to overcome salt sensitivity using Trp substitutions. Further, the property of salt resistance displayed by WLBU2 may be particularly critical to treatment of infections in diseases that may disturb the normal salt homeostasis in certain human tissues (*e. g.*, cystic fibrosis airway) (209).

The recognition that CAP activity is usually suppressed in serum has restricted the development of antimicrobial peptides mainly to topical or local applications (*e.g.*, skin, respiratory tract infections) (209, 246, 327). As described in this report, standard bacterial killing assays revealed that LL37 was totally inactivated in human serum, suggesting that, like most widely studied host-derived peptides, LL37 may not have evolved to fight systemic infections. In contrast, WLBU2 was not only highly potent against *P. aeruginosa* in human serum but also achieved complete killing by 20min, a potential advantage against rapid peptide degradation in a complete host environment. In human serum, the equilibrium between free and protein-bound peptide molecules could explain why bacterial killing is slower in comparison with killing in PBS. As more free molecules become associated with their bacterial target, the equilibrium would shift toward the progressive release of more peptide molecules, thus leading to effective but slower bacterial killing in human serum.

Once WLBU2 was proven effective in serum, it was deemed logical to investigate whether it would be as potent in human blood (in the context of biological plus host cell environments). The efficacy of WLBU2 in the bacteremic model implies that it overcame the potential inhibitory effects of physiological serum salt concentrations, including divalent cations.

In fact, it is more likely that serum proteins such as albumin or apolipoproteins (381) are responsible for the lower activity (in comparison with PBS) in human serum and whole blood. Notably, the antibacterial activity of WLBU2 was unaltered when blood samples derived from donors with hyperlipidemic disorders (milky blood or serum) were used (data not shown). Another important finding was the slightly lower (20-30%) antibacterial activity in co-culture assays in comparison to bacterial killing alone in human serum. This minor difference in activity might be due to interactions of the host cells (erythrocytes and leukocytes) with the peptide. The immunomodulatory properties of some host-derived CAPs have been demonstrated (358). Similarly, WLBU2 may have other effects on host cells, particularly on leukocytes, which is worthy of further investigation. These results provide new information that is critical to the characterization of the therapeutic potential of WLBU2 for systemic applications.

In light of all this evidence for the antibacterial efficacy of WLBU2 *in vitro*, it was important to know whether there was a chance this peptide could display any toxicity to mammalian cells after a short- or long-term exposure. Consistent with the bacteremic model, the MTT staining assays in human serum demonstrated that, given both bacterial and host cell targets, the peptide could discriminate against the bacterial cells while sparing the leukocytes and HSF cells. Likewise, a long-term treatment of these cells in the absence of bacteria had no effects on their viability. Moreover, the lymphocyte proliferation assay ruled out the concern that the peptide might specifically and adversely affect host cell functionality.

One of the greatest obstacles in the field of antimicrobial peptides is the transition from test tube to animal models because of the sensitivity of CAPs to biological environments, as demonstrated in this study. Due to these limitations, antimicrobial properties of CAPs fall short of supporting their clinical use, with a just few exceptions (*e.g.*, the polymyxins) (65, 339) .

The *in vivo* toxicity and *in vivo* efficacy of WLBU2 were also characterized using an IP model of *P. aeruginosa* bacteremia. The results indicated that WLBU2 was nontoxic at up to four times the effective therapeutic dose when administered systemically. Initially, WLBU2 was able to rescue a group of 14 mice from the progression of a *P. aeruginosa* infection. The reproducibility of these results provides convincing evidence for the systemic efficacy of WLBU2 against *P. aeruginosa*. An interesting feature of this IP infection model is the successful induction of *Pseudomonas* bacteremia without the need for an immunosuppressive drug (234, 312). This important aspect of the mouse model will render possible the characterization of the immune modulatory properties of WLBU2 *in vivo* either on its own or in the context of a *P. aeruginosa* infection in a fully immunocompetent host.

In conclusion, we have demonstrated that a *de novo* antimicrobial peptide was able to overcome the challenges of physiological serum concentrations of NaCl, Mg^{2+}, and Ca^{2+}, while the synthetic form of the human peptide LL37 displayed a high sensitivity to NaCl and divalent cations. In addition, LL37 activity was completely suppressed in human serum. In contrast, WLBU2 displayed high efficacy in human serum and the ability to eradicate a bacteremic condition *ex vivo*. Furthermore, there were no observed adverse effects on mammalian cells in terms of both cytotoxicity and functionality of blood lymphocytes. Finally, the *in vivo* efficacy of WLBU2 was demonstrated in an IP model of *P. aeruginosa* infection. These results, while promising, underscore the need for comparative studies of WLBU2 and standard antipseudomonal therapeutics (*e.g.*, colistin, tobramycin) prior to its further consideration for clinical trials. Such studies should include, but not be limited to investigating dose-dependent response to bacteremia treatments in an IV infection model, peptide pharmacokinetics, immunogenicity, and influence on cytokine levels within or outside of the setting of an infection.

3.6. ACKNOWDGEMENTS

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4. CHAPTER 4: DE NOVO-DERIVED CATIONIC ANTIMICROBIAL PEPTIDE ACTIVITY IN A MURINE MODEL OF *P. AERUGINOSA* BACTEREMIA

4.1. Abstract

Cationic antimicrobial peptides (CAPs) are a very diverse group of agents that demonstrate broad activity against Gram-positive and -negative bacteria. Because of the emergence of drug-resistant bacteria, CAPs have been extensively investigated as a potential source of new antimicrobials with novel mechanisms of action that may complement current antibiotic regimens. A major challenge to successful development of CAPs for clinical use is the suppression of antimicrobial activity under biological fluids (*e.g.*, blood). We have previously analyzed the activity and selectivity of the *de novo*-derived antimicrobial peptide WLBU2 in several biologically relevant conditions. Recently, we demonstrated potent activity of WLBU2 in an intraperitoneal mouse model of *Pseudomonas aeruginosa* infection. In this study, we first evaluated the influence of WLBU2 on inflammatory cytokines to further characterize the toxic property of the peptide. The data indicate that WLBU2 had no effect on the levels of most cytokines, except for a minor stimulatory effect on IL1-β and TNF-α, the most potent inflammatory cytokines. We then characterized the *in vivo* efficacy of this peptide in an intravenous infection model. WLBU2 (3 to 4mg/kg) not only protected mice prophylactically, but was highly therapeutic when administered IV to *P. aeruginosa*-infected animals, with complete elimination of the bacteria from the blood and other tissues. These results, together with our previous data on the toxic potential and efficacy of WLBU2 in the IP infection model, provide strong evidence for a potential application of this peptide in the treatment of septicemia.

4.2. Introduction

Despite the development of safe and potent antibiotics, bacterial diseases remain a worldwide health crisis. A serious concern is the emergence of multiple drug resistance (117, 130, 137, 211, 223, 341). For instance, *Pseudomonas aeruginosa* is a gram negative bacterium known to cause disease in immunocompromised patients. Although opportunistic, *P. aeruginosa* poses a serious burden to the clinical management of infectious diseases because of its ubiquity, frequency of transmission in hospitalized patients, and high propensity to become resistant to multiple antibiotics. *P. aeruginosa* can cause severe diseases in patients with cystic fibrosis, burns, ventilator-associated pneumonia, and septic infections (53, 126, 127, 144, 169, 253, 350, 392). Therefore, there is a critical need to develop more effective antimicrobials with novel bactericidal mechanisms to circumvent the obstacle of multiple drug resistance.

A potential source of novel therapeutics are antimicrobial peptides referred to as cationic amphipathic peptides or CAPs (360, 442-444). The diversity of the primary sequences of these peptides and the conservation of amphipathicity in the native (α-helix, β-sheets, loops, *etc.*) structure suggest an essential role of the amphipathic property in antibacterial activity. Based on this amphipathic motif and amino acid compositions, CAPs generally demonstrate broad environment-dependent activity against Gram-positive and -negative bacteria (244, 360). Stored in phagocytic granules and secreted by epithelial cells in response to the presence of microbial organisms, host antimicrobial peptides play an essential role in innate immunity by providing an important mechanism for rapid and efficient clearance of invading pathogens (79, 80, 360). Some CAPs display modulation of cytokine levels and the adaptive immune response (360).

Two critical issues on the functioning of CAPs are the suppression of activity in biological fluids (*e.g.*, blood or blood-derived matrices) and the environment-dependent host

toxicity of many antimicrobial peptides. Numerous studies have been conducted to address these two issues by adopting two approaches. One is to modify natural peptides to improve their potency, and the other to design CAPs *de novo* based on structure-function specificity (162, 186, 252, 286, 309, 400). It was previously shown that it was possible to achieve optimal potency in phosphate buffered isotonic NaCl by designing a 24-mer amphipathic peptide (WLBU2) with Arg on the hydrophilic face and mainly Val and 3 Trp residues in the hydrophobic face (162). More recently, we demonstrated that WLBU2 was highly active against *P. aeruginosa* in human serum and whole blood. To characterize the selective property of WLBU2 under these conditions, a competition assay was developed, which consists of bacteria and human cells simultaneously presented to the peptide under the aforementioned conditions. The results of these studies indicated that WLBU2 was nontoxic to red and white blood cells with a therapeutic window of >30μM. What remained unclear from these investigations was the potential *in vivo* efficacy of WLBU2 against *P. aeruginosa*.

We hypothesized that the systemic administration of this peptide would prevent the progression of *P. aeruginosa* infections in experimental mouse models. This hypothesis was partially addressed in a previous report in which we described the efficacy of WLBU2 against *P. aeruginosa* in an intraperitoneal (IP) mouse model. In the current study the prophylactic, therapeutic, and cytokine modulatory properties of this peptide were investigated in an intravenous (IV) model of *P. aeruginosa* infection. It was found that WLBU2 was only weakly stimulatory to inflammatory cytokines, and that systemic administrations of WLBU2 increased survival of *P. aeruginosa*-infected mice by preventing the progression of the bacterial disease.

4.3. Materials and Methods

4.3.1. Organisms

The laboratory pathogen *Pseudomonas aeruginosa* PAO1 was used as described previously. The mouse of choice was the female Swiss Webster mouse (25-30g) strain, which was purchased from Taconic (Germantown, New York) because it was demonstrated that this strain can be susceptible to *P. aeruginosa* bacteremia without the use of an immunosuppressant. All animals were maintained and procedures performed according to protocol approved by the Institutional Animal Care & Use Committee (IACUC animal protocol number 0402610A-1) of the University of Pittsburgh, Pittsburgh, PA. All mice were housed in individual cages under constant temperature (22°C) and humidity using a 12-hr light/dark cycle.

4.3.2. Peptide Synthesis

The engineered peptide derivative WLBU2 (RRWVRRVRRWVRRVVRVVRRWVRR) was synthesized using standard FMOC synthesis protocols as previously described (394, 396). The synthetic peptide was characterized and purified by reverse-phase HPLC on Vydac C18 or C4 columns (The Separations Group, Hesperia, CA), and the identity of each established by mass spectrometry (Electrospray Quatro II triple quadruple mass spectrometer, Micromass Inc., Manchester, UK). Peptide concentrations were determined using a quantitative ninhydrin assay as previously described. A peptide sample of known concentration was used to evaluate several stocks of WLBU2 by spectrophotometric analysis, based on Trp absorbance at 280nm.

4.3.3. Evaluation of Inflammatory Responses

To evaluate the influence of WLBU2 on inflammatory cytokine levels, mice were treated IV with WLBU2 (1 and 3 mg/kg), heat-killed bacteria (10^8cfu), or PBS. Blood was collected at 3h and 5h, and sera isolated for quantification of an extensive panel of cytokines (IL1-β, TNF-α, Rantes, MIP-1α, etc.) using the Bio-Plex protein array system (Bio-Rad, Hercules, Calif.) according to the manufacturer's instructions. The Bio-Plex Manager version 3.0 software (Bio-Rad) was used to determine the concentration (picograms per mL) of each cytokine or chemokine. Data are expressed as fold increase by dividing the cytokine concentration for each test sample to that of the control (PBS-treated mice).

4.3.4. Intravenous Bacterial Inoculation Followed by Intravenous Antibacterial Therapy

Suspensions of mid-log phase bacteria were centrifuged at 2000 × *g* for 10 min using the Adams Dynac I centrifuge (Becton Dickinson, Franklin Lakes, NJ). Supernatants were discarded, and the bacteria resuspended and diluted in sterile PBS to achieve a concentration of approximately 2-4 × 10^8 colony-forming units (cfu)/mL. Mice were injected IV (via the tail vein) with 0.1 mL of the bacterial suspensions (2-4 x 10^7cfu, the minimum lethal dose). The animals were then randomized to receive IV isotonic sodium chloride solution (control group), or 1, 1.5, 3, and 4mg/kg WLBU2 approximately 60min after bacterial challenge. The animals in each group, which included 7-11 mice, were returned to individual cages and subsequently monitored for up to 7-10d for survival. The end points of the study were indicated either by 7-10d survival or by complete absence of motility as a sign of terminal illness.

4.3.5. Evaluation of Treatment

Quantitative blood cultures on TSA plates were performed to determine bacterial loads over the course of the infection. Blood samples were obtained from the tail vein by aseptic percutaneous puncture 1h to 24h after bacterial challenge and serially diluted. Then, a 0.1-mL volume of each dilution was spread on TSA plates and incubated at 37°C overnight for enumeration of developed colonies. Finally, toxicity was evaluated on the basis of the presence of drug-related adverse effects such as signs of inflammation, weight loss, and presence of bacteria in the blood and tissues. Throughout the course of the infection or at the disease endpoint, animals were euthanized, and tissues weighed and homogenized using 70µm cell strainers (Becton Dickinson, Franklin Lakes, NJ) to determine bacterial cfu/g tissue.

4.3.6. Statistical Analysis

Data were analyzed using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com). Kaplan-Meir survival analysis was performed, and the log-rank test used to compare survival between groups. Significance was accepted at a P value of <0.05.

4.4. RESULTS

4.4.1. *In Vivo* Toxicity

To characterize the *in vivo* efficacy of WLBU2, it was important to examine its potential *in vivo* toxicity within a range of concentrations to determine the maximum tolerated dose (MTD). We previously described the potential toxicity of WLBU2 in mice when systemically

administered. It was found that the maximum tolerated dose of WLBU2, the highest IV dose of WLBU2 that caused no obvious toxicity to the mice, was 12mg/kg (Table 5). Of note, no

Table 5. WLBU2 is less toxic than other comparable antimicrobial peptides [a]

MTD (mg/kg body weight)	
	Mice (IV)
WLBU2	12
Colistin	<5
K_6L_9	<6

[a]In three independent studies mice were injected IV with WLBU2, colistin (antimicrobial peptide used clinically against Gram-negative bacteria), or K6L9 (antimicrobial peptide in development, with 33% D-amino acid content). The maximum tolerated dose (MTD) of WLBU2 (12mg/kg) is more than two-fold that of colistin (<5mg/kg) and K_6L_9.

histopathologic changes were observed within 3-5h or 10-21d post-treatment (data not shown). As indicated in Table 5, WLBU2 compares favorably with other peptides (colistin and K_6L_9) that have been tested for toxicity in animal models. Colistin is an antimicrobial peptide that is used clinically in complicated cases of Gram-negative infections; whereas K_6L_9 (33% D-amino acid content) is a novel CAP that demonstrates significant efficacy in bacteria-infected mice (91). The MTD of either colistin or K_6L_9 is half that of WLBU2.

Inflammation-induced tissue injury is another measure of host toxicity. Many host-derived antimicrobial peptides have the property of upregulating cytokine levels. Although cytokines may be helpful in alerting key cellular components of the immune system for neutralizing infections, inflammatory cytokines often also cause tissue injury and death. Antimicrobial peptides may modulate the levels of several potent inflammatory cytokines such as IL-1, IL-6, IL-8, and others leading to tissue injury (413, 414, 432). Hence, we attempted to characterize the cytokine modulatory property of WLBU2 by comparing cytokine levels in mice treated either with heat-killed bacteria or with different doses of WLBU2 after 3h and 5h. As

shown in Figure 12A and 12B, the levels of inflammatory cytokines in mice treated with heat-killed bacteria (HKB) were markedly elevated reaching up to 15 (IL1-β) and 32 (TNF-α) times the basal level (control). In contrast, the cytokine levels in WLBU2 (1 or 3mg/kg)-treated mice were less than two-fold the basal level (PBS-treated mice) at 3h (Figure 12A and 12B). After 5h post-treatment, cytokine levels in WLBU2-treated mice were reduced to basal levels, whereas only a partial reduction was observed for HBK-injected mice. These results indicate that WLBU2 did not display significant inflammatory property during the first 5h following systemic administration.

4.4.2. In Vivo Efficacy

4.4.2.1. *Influence on Survival*

We previously described the efficacy of WLBU2 against *P. aeruginosa* in an intraperitoneal (IP) infection model. The advantage of this model is the induction of a successful infection without the need for immune suppression. Mice receiving WLBU2 (2.4mg/kg) were all protected against bacterial disease. In this IP model, however, it was not clear whether the increased survival observed in WLBU2-treated mice (compared with mock-treated mice) was due either to the prevention of bacteremia and septicemia or to the elimination of *P. aeruginosa* infections localized in the internal organs. Hence, we sought to further characterize the *in vivo* efficacy of WLBU2 in an IV infection model.

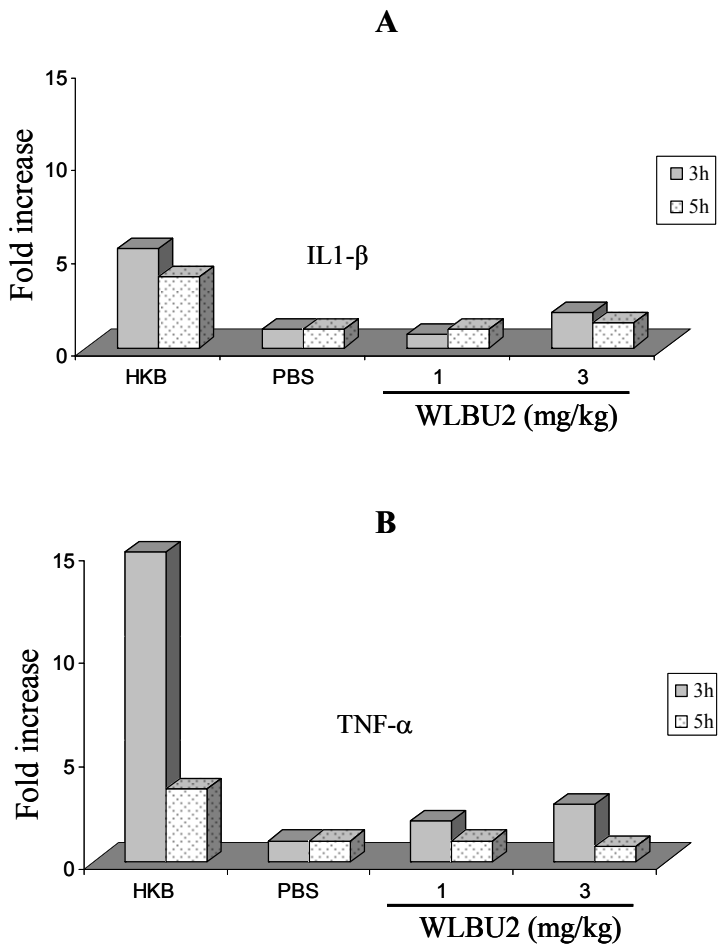

Figure 12. Influence of WLBU2 on Inflammatory Cytokines

To determine the cytokine modulatory property of WLBU2, mice were treated with either PBS, heat-killed bacteria (HKB), and 1, or 3mg/kg WLBU2. Mice were sacrificed at 3 and 5h and blood samples collected for serum isolation and cytokine quantification as described in Materials and Methods. Potent inflammatory responses occurred in mice injected IV with heat-killed bacteria. In contrast, WLBU2 had a minor effect on cytokine levels at 3h post-treatment, which was reduced to negligible levels by 5h.

First, the minimum IV PLD (*Pseudomonas* lethal dose), the lowest IV bacterial dose (2-3 x 10^7 cfu) leading to 100% mortality in 24-30g mice, was determined (data not shown). To evaluate the prophylactic effects of WLBU2, the IV PLD was administered to 6 mice 1h after IV injection of the peptide. Also included in this experiment are a control (placebo) group (11 mice)

receiving isotonic NaCl (PBS) IV 1h post-infection and a therapeutic group (11 mice) given 3mg/kg WLBU2 IV 1h after bacterial injections (Figure 13). In comparison with the mock-treated mice, no lethality was observed in WLBU2-treated groups. Statistical analyses using the log-rank test revealed a P value of <0.0001. These results provide strong evidence for a potential prophylactic and therapeutic role of WLBU2 in the treatment of *P. aeruginosa* infections.

The convincing reproducibility of these data is suggested by the large number of mice (up to 11 per group) used; however, it remains unclear how these infected mice would respond to

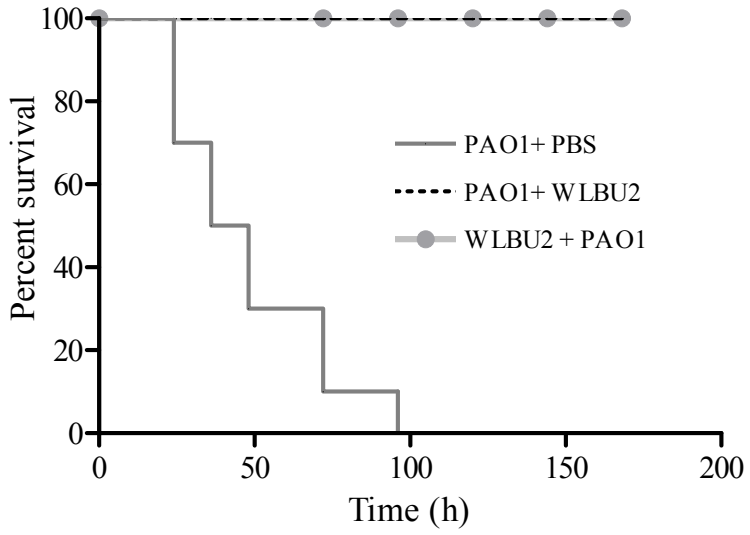

Figure 13. WLBU2 protected mice infected IV with *P. aeruginosa*

To characterize the *in vivo efficacy* of WLBU2, we developed an IV infection model (both PAO1 and WLBU2 administered IV). After administration of an IV PLD (3-4 x 10^7 cfu), 11 mice were treated with either 3 mg/kg WLBU2 (PAO1 + WLBU2) or PBS at least 1h post-infection. At the experimental endpoint, 100% survival was observed for WLBU2-treated mice and no bacterial cells were recovered from the blood when compared with 10^8-10^9 cfu/ml blood recovered in mock-treated mice (data not shown). A log-rank test reveals a P value of <0.0001 when comparing survival of WLBU2-treated with that of mock-treated mice.

variable doses of this peptide. To address this issue, *P. aeruginosa*-infected mice (9 mice/group) were treated IV with varying doses (1, 1.5, 3, and 4mg/kg) of peptide 1h post-infection. As shown by the Kaplan-Meir survival analysis in Figure 14A, 90% lethality was observed for mice

treated with 1.5mg/kg, and the 1mg/kg WLBU2-treated group displayed 100% mortality. In contrast, 90 and 100% survival occurred in mice treated systemically with 3 and 4mg/kg, respectively. Although the mouse immune system is suspected to play a role in eradicating the

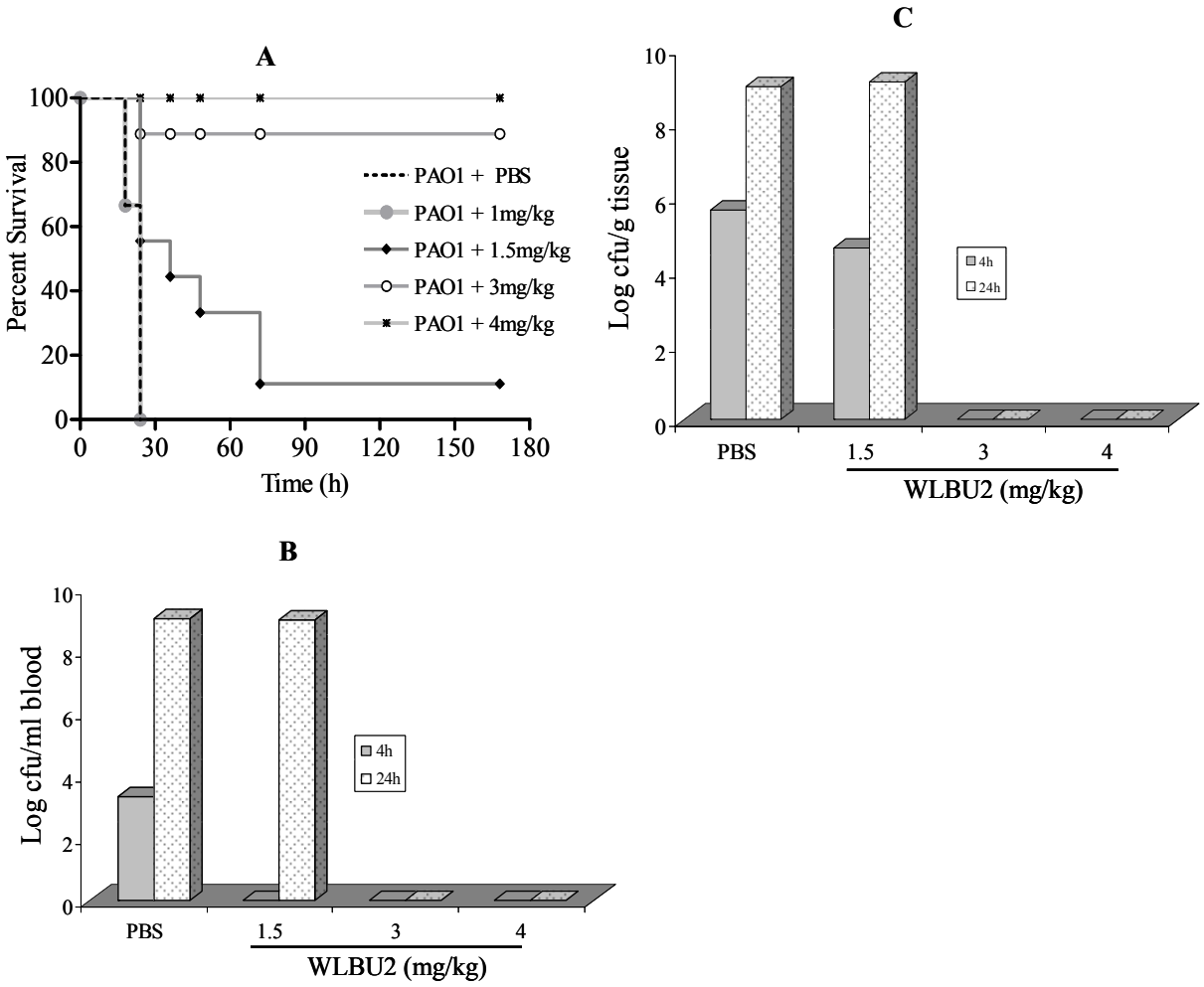

Figure 14. Survival and bacterial loads vary with peptide doses

To evaluate the influence of WLBU2 on survival at varying doses, infected mice were treated with 1, 1.5, 3, and 4mg/kg peptide and followed for up to 7d post-treatment. WLBU2 effectively eradicated the infection at a minimum of 3mg/kg (A), with 1 case of fatality of 9 mice treated. Mice were also euthanized at 4 and 24h to determine bacterial loads as described in Materials and Methods. Bacterial loads increased from 10^3 (blood) or ~10^5 (kidney) to 10^9 cfu per mL blood or g tissue in PBS-treated mice. The bacteria were completely eliminated from the blood and tissues by 4h in mice injected with either 3mg/kg or 4mg/kg WLBU2, which was consistent with survival.

infection, the efficacy of WLBU2 is strongly indicated and solely accounts for the difference in survival between the control and experimental groups.

4.4.2.2. *Influence on bacterial loads*

During the course of the infection, it was important to evaluate the progression of bacterial growth in blood and tissues. Following the administration of a minimum PLD of 2-3x 10^7cfu, bacterial loads in the blood initially decreased during the first 3-4h of infection. Typically after a dose of 2-3 x 10^7cfu, the bacterial load in systemic circulation was progressively reduced from 10^6cfu/ml 1h post-infection (not shown) to approximately 10^3cfu/ml after 4h post-infection (Figure 14B). This initial control of bacterial load in the blood of mock-treated mice, however, is dependent on the initial dose. In a few cases, PBS-treated mice that were given approximately 1.5-2x10^7cfu successfully eliminated the bacteremia while the infection remained localized in the organs before the progression to weight loss and terminal disease within 3-4d post-infection (data not shown). Usually in the early phase of infection, bacteria were able to invade the organs with bacterial loads varying between 10^4 and 10^5cfu/g of tissue in animals treated with either PBS or insufficient doses of peptide (1-1.5mg/kg). In contrast, no bacteria were found in the blood (Figure 14B). and in the organs of mice treated with 3-4mg/kg WLBU2 1h post-infection (Figure 14C). Consistent with our survival analysis, bacterial loads in the organs and blood of mice treated with 1-1.5mg/kg after 24h were comparable to those in mice treated with PBS. Within 16-24h, signs of terminal illness (lack of motility and hypothermia) began to appear, and bacterial loads reached ~10^9 cfu per mL blood or per gram tissue (Figure 14C). These results suggest that WLBU2 plays an important role in helping to control the bacterial infection in peptide-treated mice. However, the efficacy of the peptide may be complemented by the mouse innate immunity to completely eliminate the bacterial infection. To better understand the

significance of these results, we compared the independently determined therapeutic indices of WLBU2 and K_6L_9 against *P. aeruginosa*. In respective models, WLBU2 displayed a higher activity (95-100% survival) *in vivo* than the D-enantiomer of K_6L_9 (75% survival) (Table 6).

Table 6. WLBU2 compares favorably with other antibacterial peptides for *in vivo* efficacy [a]

	Mice	Bacterial strain	Bacterial dose (cfu)	Route of infection	Route of treatment	Peptide dose (mg/kg)	number of animals	Survival
WLBU2	Swiss Webster	PAO1	10^7	IP	IV	2.4	14	14
			3×10^7	IV	IV	3	20	19
K_6L_9	CD1	ATCC 27853	10^6	*NS	IV	**6	8	6

[a]The peptide K_6L_9, the newest CAP to demonstrate in vivo efficacy against P. aeruginosa, cured 6 of 8 mice following two equal doses of 3mg/kg as previously described. WLBU2 cured 14/14 mice in the IP and 19/20 in the IV infection model at a single dose of 3mg/kg. The bacterial infection was completely eradicated within 4 to 24h, which was consistent with no sign of disease. *not stated in report; **2 doses of 3mg/kg daily

4.5. Discussion

We demonstrated that the *de novo*-derived peptide WLBU2 was highly active against *P. aeruginosa in vivo*. This study was initiated after the observation of WLBU2 activity in human blood and serum. First, we evaluated the *in vivo* toxicity of WLBU2 and showed that mice treated with up to 12mg peptide per kg body weight displayed no apparent signs of toxicity. We then described the therapeutic efficacy of WLBU2 in an IP infection model. In the current study, we demonstrated that WLBU2 was highly prophylactic and therapeutic in an IV infection model in a dose-dependent manner. Finally, WLBU2 displayed a slight and transient stimulation of some inflammatory cytokines, namely IL1-β and TNF-α.

The role of antimicrobial peptides in innate immunity has been long established through various studies of the microbial killing mechanisms of neutrophils and macrophages (449, 450, 452). However, it was not until the last two decades that CAPs have been seriously considered as a potential therapeutic source because of the increasing awareness of the problem of multiple drug resistance (90, 257, 280). Although numerous CAPs with potent antibacterial efficacy *in vitro* have been identified or engineered, very few have displayed significant activity in animal models (91, 312). An important explanation is the suppression of activity in biological environments. To address this issue, a series of studies have been initiated in our laboratory to develop *de novo* CAPs for clinical applications. From these studies, we have demonstrated that the peptide WLBU2 retained its activity against *P. aeruginosa* in the presence of physiological concentrations of monovalent and divalent cations, human serum, and whole blood. These studies warranted further investigations of WLBU2 efficacy *in vivo*.

The IP mouse model is one of the most common animal models for testing antibacterial efficacy *in vivo*. Since mice tend to be resistant to chronic *P. aeruginosa* infection, it is a common practice to induce *P. aeruginosa* dissemination by suppressing the mouse innate immunity prior to IP injection of the bacteria (312). Although the *in vivo* efficacy of a few CAPs is evaluated in this model, we found this system inappropriate for our study. An important reason is that some antimicrobial peptides demonstrate cytokine modulation. We thought that some other properties of WLBU2 that could influence the outcome of an infection in the context of a functional immune system would have been masked by artificially inducing immune suppression. Thus, we were able to successfully develop an IP infection model with a *P. aeruginosa* dose of 10^7cfu, which is approximately ten times the bacterial dose commonly used with concurrent immune suppression (173, 233). In this model with a single dose of 2.4mg/kg

administered IV, WLBU2 was able to eliminate the bacteria from the organs and the blood and protected all mice from the progression of an acute infection as previously described. However, it was not clear whether the peptide was protecting the mouse by sterilizing the systemic circulation or by eradicating the initially localized infections. Further, bacteremia is more realistic than peritoneal infections in a clinical situation. Thus, we sought to further investigate the *in vivo* efficacy of WLBU2 in an IV infection model.

We predicted that the induction of an IV infection would require a lower dose of bacteria than that of an IP infection. To our surprise, we had to triple the IP PLD to induce a successful IV infection. Infections localized in the internal organs evidently occurred rapidly after the IP injection of *P. aeruginosa*. In contrast, in the IV infection model the direct exposure of bacteria to most of the circulating immune cells was likely to result in effective and rapid clearance of bacteria from the blood prior to or during dissemination to the organs. This is consistent with the concomitant reduction of bacterial load in the blood and increase of bacterial cfu in the organs during the first few hours of infection in mock-treated mice. An insufficient dose of WLBU2 (*e.g.*, 1.5mg/kg) resulted in a transient control of initial bacteremia but not of infections localized in the tissues. Evidently, the localized infections served as a new source of bacteria for invasion of the systemic circulation leading to death by 36-48h. This explanation is further supported by the complete absence of bacteremia in untreated (no peptide) mice injected (IV) with only $< 2 \times 10^7$cfu 3-4 days prior to terminal disease. This finding was observed during the determination of the IV PLD (data not shown).

After establishing the IV infection model, the prophylactic potential of WLBU2 (3mg/kg) was successfully demonstrated, with the complete prevention of infection. This finding warrants further investigation for possible prophylactic antimicrobial application prior to certain surgical

procedures. It also provides evidence for peptide stability during at least 1h in systemic circulation. Consistent with this observation were the elimination of all bacteria in 11 *P. aeruginosa*-infected mice (in the presence of 3-4mg/kg peptide) and the dose-dependent effect of WLBU2 on survival. It was shown that WLBU2 was subtherapeutic at a dose of 1.5mg/kg or lower. In contrast, 19 of 20 mice were protected from *P. aeruginosa* infection by a single IV dose of 3mg/kg, and 9 of 9 mice by 4mg/kg. The success of our *in vivo* models is a remarkable advancement in the field of antimicrobial peptide research as indicated by the comparison of the efficacy of the peptide K_6L_9 with that of WLBU2 in Table 6. Our data are not only statistically significant (P values <0.0001), but the high number of mice used in comparison with what is observed in other in *vivo* studies is also supportive of the reproducibility of these data (91).

In a recent study, we characterized the *in vivo* toxicity of WLBU2 and determined a maximum sublethal dose of 12mg/kg. What remained unclear was whether WLBU2 could potentially cause tissue injury by upregulating the levels of potent inflammatory cytokines, a property of some antimicrobial peptides (24, 432). Judging by the important role of cytokines in host defense, there are obviously some benefits to cytokine stimulation by natural CAPs. However, during the progression of bacterial disease, the stimulation of potent inflammatory responses is generally undesired for the sake of preserving tissue integrity and preventing disseminated intravascular coagulation. Thus, from a clinical standpoint the anti-inflammatory property of an antimicrobial drug is preferred. It is from this perspective that we tested the influence of WLBU2 on cytokine levels. From an extensive panel of cytokines analyzed, IL1-β and TNF-α were given more attention because of their importance in systemic inflammation and the notable changes in their levels compared to other cytokines. WLBU2 displayed only a minor stimulation of these two cytokines. These results demonstrated that the inflammatory property of

WLBU2 would not be a concern in its eventual use against clinical diseases caused by *P. aeruginosa*.

Taken together, our data demonstrate the successful development of a *P. aeruginosa* bacteremic model in the context of a competent immune system. Using this model, we were able to show that the *de novo*-derived CAP was highly prophylactic and therapeutic against *P. aeruginosa* bacteremia in mice. WLBU2 compares favorably with the standard antimicrobial peptide colistin and the novel CAP K_6L_9 (91). These results provide fundamental information that may be useful for evaluating *in vivo* efficacy of other CAPs. Further, the data underscore the need for comparative studies of WLBU2 and standard antimicrobials in similar mouse models to establish a potential for clinical trials.

4.6. ACKNOWLEDGEMENTS

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5. CHAPTER 5: Overall Discussion

5.1. Overall Summary

The ubiquity of antimicrobial peptides and their role in host immunity suggest that they could be used as an additional source of agents with novel antimicrobial mechanisms against MDR pathogens. Despite extensive efforts to develop CAPs for clinical use, the preservation of CAP activity in biological environments remains a tremendous challenge. In the first aim, we demonstrated the design of antimicrobial peptides with potent antibacterial activity based on a cationic amphipathic motif (LBU) by using solely Arg on the cationic face and Val residues on the hydrophobic face. Antibacterial activity positively correlated with helicity and length, but no net gain in activity was observed for peptides longer than 24 residues. Not surprisingly, the inclusion of Trp residues on the hydrophobic face improved the activity of the 24-mer LBU2 as predicted. The LBU derivative WLBU2 was identified as the lead compound and retained optimal active against a battery of 16 clinical isolates of *P. aeruginosa*.

The second objective was to compare the *in vitro* efficacy of the natural antimicrobial peptide LL37 with that of WLBU2 under biologically relevant conditions. The activity of LL37 was consistently suppressed in the presence of increasing concentrations of Mg^{2+} and Ca^{2+} and in human serum. In contrast, WLBU2 activity was refractory to physiological concentrations of these cations. A remarkable finding was the complete killing of *P. aeruginosa* by WLBU2 in human serum and whole blood, without adverse effects on host cells. These results formed the basis for evaluating the efficacy of WLBU2 in experimental mouse models.

The final aim was to investigate the *in vivo* efficacy of WLBU2. First, we determined the MTD of the peptide to identify a range of potential therapeutic doses. In the IP infection model,

we established an effective therapeutic dose of 2.4mg/kg body weight (1/6 MTD). Then we were able to show that WLBU2 was both prophylactic and therapeutic in an IV infection model in a dose-dependent manner. Furthermore, WLBU2 only slightly stimulated the inflammatory cytokines IL1-β and TNF-α.

5.2. Peptide Design

To develop potent antimicrobial peptides, two main approaches have been used. One is to evaluate host-derived antimicrobial peptides for a better understanding of the structural determinants of biological activity and for improved potency (394, 396). The other is to apply the lessons derived from these studies to *de novo* engineering of CAPs with higher antibacterial activity (286, 365, 400). Studies of several CAPs identified some fundamental structure-function specificities. Antimicrobial peptides are typically short (10-50 amino acid residues), and they all fold into amphipathic structures in the presence of lipid membranes or membrane mimetic environments (48, 59, 443, 444). Although a consensus sequence has not been identified, specific amino acids may be associated with antimicrobial activity. Either Arg or Lys (or both) is commonly found in the hydrophilic domains of natural CAPs (178, 352, 353). The hydrophobic amino acids Val (*e.g.*, LLP1), and Trp (*e.g.*, indolicidin), and few others are also common (9, 10, 226, 352, 353, 394, 416). Various studies indicate that selective toxicity is maintained by a balance between cationicity and the level of hydrophobicity. While increasing cationicity may enhance selective toxicity against Gram-negative organisms, raising the level of hydrophobicity may have a greater impact on Gram-positive bacteria and increase host toxicity (153, 154, 189, 217, 307, 359, 425). Consistent with these studies is the increased potency of LLP1 derivatives with higher Arg and Val content and Trp substitution (330).

An important consideration in peptide design is the minimum length required for optimal antimicrobial activity to minimize the cost of production. McLaughlin and co-workers initiated the concept of multimeric peptide design from a unique sequence(251). Although most natural peptides are short (usually <50 amino acids), it is not possible to predict the minimum length for optimal activity based on this observation alone, and natural peptides do not necessarily require the membrane spanning length of 22 residues for activity. Thus, it was necessary to adopt an approach consisting of varying length without considerably changing the primary sequence. There were a few studies of *de novo* engineered peptides demonstrating activity at 20 amino acids or less, but most of these were not comparative studies based on length variation (252, 286, 400). McLaughlin and colleagues were able to generate a multimeric series of peptides based on a 7-residue primary sequence (252). However, a maximum length of only 21 residues was used and no conclusion could be drawn about longer peptides of similar sequence. Thus, we decided to extend this multimeric concept to 48 residues based on an LBU length of 12 residues. The amino acids Arg and Val were used due to their large proportion in LLP1, and Trp residues were substituted at a intervals of at least 7 amino acids to avoid disrupting the predicted helical structure (396). Using a helical wheel analysis for the generation of idealized amphipathic helices, the LBU peptides were engineered with Arg and Val maximally segregated into hydrophilic and hydrophobic faces, respectively. Given the membrane-seeking property of Trp, it was possible to predict an improvement in potency with Trp-containing peptides (354). Hence the WLBU peptides were derived from the parent LBUs by Trp substitution.

5.3. Structural Determinants of Antimicrobial Activity

Length & helicity: Prior to the design of the LBU and WLBU series, only few engineered peptides with antimicrobial activity were reported. Whether the focus was on cyclic or linear CAPs, the amphipathic motif was a common feature, but the influence of length on activity remained unclear (68, 286, 447). Javadpour *et al*. demonstrated a positive correlation between length and activity using the aforementioned multimeric peptide series, but they limited the maximum length to 21 residues (252). Similar to this study, our data have shown a positive correlation between length and activity. However, by extending the length of the LBU peptides to 48 residues, we were able to demonstrate that optimal activity could be achieved at 24 residues. We predicted that the 48-mer would be by far the most potent based on the fact that charge and hydrophobicity increase with length. Contrary to this hypothesis, we found that the cationic and hydrophobic densities (rather than the overall charge and hydrophobicity), coupled with an optimal membrane spanning region (>22 residues), account for the optimal activity achieved by LBU2.

The helical propensity of the peptides also had a major influence on activity. Expanding the peptides from 12 to 24 residues more than tripled the helicity. Studies of interactions of antimicrobial peptides with model membranes indicate that induction of the native conformation of a particular CAP is essential to pore-forming or cell penetration activity (178, 250, 326, 352, 369, 372, 373, 416, 422). A recent study by Park et al. suggests that the disruption of the helical structure suppresses activity. It was shown that stabilizing the helical structure with a helix-capping motif significantly enhanced activity in the presence of high salt concentrations (327). Furthermore, several studies demonstrate that CAPs interact more strongly with negatively charged than with zwitterionic model membranes (8, 11, 55, 73, 74, 145, 160, 444). Considering

that LBU2 has the strongest cationic density (13 + charges over a total of 24 residues), it is not surprising that optimal antimicrobial activity was achieved at 24 residues.

Trp & activity: Similar to the relationship between activity, length, and helicity in the LBU series, the 24-mer WLBU was the most potent of the WLBU peptides. It has the same cationic density, amphipathicity, and helical propensity as LBU2. But the three Trp residues in the hydrophobic face considerably enhanced the activity of WLBU2 against *S. aureus* in the presence or absence of salt while maintaining optimal efficacy against *P. aeruginosa*. Circular dichroism analysis reveals no change in the helical structure of WLBU2 compared with that of the parent LBU2. The importance of Trp in CAPs is supported by other reports. For instance, Vogel and others have shown that Trp and Arg residues in lactoferricin or indolicidin are essential to antimicrobial activity. Other evidence is also provided by studies of engineered Arg- and Trp-containing peptides (179, 256, 352, 353, 416). The antistaphylococcal activity of LBU2 was suppressed in the presence of 150mM NaCl. In contrast, WLBU2 displayed potent activity against both Gram-negative and –positive bacteria in the presence of physiological concentrations of monovalent and divalent cations. Several studies support these differences between the two peptides. For instance, some CAPs display more affinity for the LPS-containing bacterial membrane than for Gram-positive membrane (99, 189, 190, 228, 266, 307, 386, 387, 418, 419, 421, 426). Similarly, in the presence of salt LBU2 is able to bind strongly to the negatively charged LPS. However, at the same concentration NaCl disrupts this interaction with the less negatively charged surface of *S. aureus*. Under this condition, it's highly likely that LBU2 adopts a random coil conformation. The inclusion of 3 Trp residues in the hydrophobic face of LBU2 results in higher membrane affinity. Park et al. demonstrated that a helix capping motif conferred helical stability and preservation of activity in high salt environment. Similarly,

a salt environment, being less ideal to a Trp-containing peptide, should favor the native helical conformation in the presence of a lipid bilayer (11, 116, 188, 255, 267). Thus, WLBU2 displayed potent efficacy against both *S. aureus* and *P. aeruginosa*, and salt sensitivity may be overcome by using Trp substitution in pre-existing antimicrobial peptides.

5.3.1. Structural Determinants of Selective Toxicity

Another challenge to the development of CAPs for clinical use is the suppression of activity in human serum (152). The discovery of the magainins and their broad antibacterial activity made magainin derivatives very promising as prospective clinical agents. However, the lack of activity of magainin 2 against *P. aeruginosa* bacteremia in mice and other findings that were consistent with this observation shifted CAP development toward topical applications (205, 339). To date, there have been few reports on CAP activity in biological fluids. In 2004, when the data on the activity of WLBU2 in human blood were not yet published, a group of investigators reported the activity of platelet-derived peptides in human blood and blood-derived matrices (393, 434, 436). Two years prior to that report, we designed a series of experiments to examine the selective property of WLBU2 in biological fluids. The standard RBC lysis assay in PBS was too artificial to predict the behavior of WLBU2 in a real drug-pathogen interaction. Further, bacterial and host toxicity assays performed independently cannot necessarily evaluate the selective property of a drug in the context of an infection. Thus, we developed the concept of selective toxicity in the context of a competition between the host and the pathogen co-existing either in human blood or serum. We were able to show that the activity of the human synthetic peptide LL37 was markedly suppressed in human serum and whole blood. In contrast, WLBU2 achieved complete killing in serum by 20min and was selectively toxic toward *P. aeruginosa* in

blood and in a co-culture of *P. aeruginosa* and human monocytes. These results ultimately led to the systemic evaluation of the efficacy of WLBU2 in experimental mouse models.

5.4. Progress toward Systemic Application of WLBU2

Because of limitations previously mentioned, there have been few published reports on the *in vivo* efficacy of antimicrobial peptides. One recent study describes the antipseudomonal activity of the peptide dermaseptin S4 in the murine peritoneal space; however, this was just a small advancement over a test tube model (312). To test the in *vivo* efficacy of the de novo-derived peptide WLBU2, we developed an IP-induced bacteremic model and demonstrated potent antipseudomonal efficacy of WLBU2 at 2.4mg/kg. To protect 14 mice with a single dose IV was an exceptional advancement in the field of antimicrobial peptide research.

The lack of reports on the systemic use of CAPs is not indicative of a lack of *in vivo* studies. It is likely that unsuccessful attempts to demonstrate the *in vivo* activity of engineered CAPs were reported. One possible reason for this lack of success is the short half life of antimicrobial peptides. This issue was addressed in a recent study of a CAP containing 33% D-amino acids (91). After displaying potent activity over the ineffective L-enantiomer in the presence of human serum, this peptide (injected IV) was shown to cure 6 of 8 (75%) *P. aeruginosa*-infected mice. We addressed the issue of a short half life in a different way. We demonstrated that WLBU2 (3mg/kg) was able to protect Swiss Webster mice against the development of bacteremia when administered IV 1h prior to bacterial injection. This finding suggests that the L-enantiomer of WLBU2 remained active in the murine circulatory system for at least 1h post-treatment. The efficacy of the L-enantiomer will reduce cost by eliminating the

need for the more expensive D-amino acids. Further, WLBU2 could be explored as a prophylactic agent in procedures with high risk for bacterial infection.

In our dose-dependent survival analysis, we demonstrated a minimum therapeutic dose of 3mg/kg. A total of 19/20 and 9/9 mice were successfully cured by 3mg/kg and 4mg/kg WLBU2, respectively; this is about 1/5 to 1/4 of the MTD. It is important to note that the previously tested peptides have a narrower therapeutic window. For instance, the CAP K_6L_9 gave rise to 75% survival with 2 doses of 3mg/kg. Evidently, a single dose would be nontherapeutic and the peptide is toxic at higher doses.

Together with the low stimulatory effect on inflammatory cytokines, the *in vivo* efficacy of WLBU2 in the IP and IV infection models underlines the need to further investigate WLBU2 as a potential therapeutic against *P. aeruginosa* infections.

5.5. Future Directions

Our study of *de novo* antimicrobial peptides has provided many answers, but several issues still need to be addressed in the areas of peptide design and progress toward clinical applications.

5.5.1. Peptide Design and Evaluation

Based on the literature on antimicrobial peptides, our data indicate that WLBU2 is the most extensively characterized and the most potent *de novo*-derived CAP. However, the lessons derived from the development of WLBU2 could be used to generate CAPs with greater therapeutic indices. We now have a better understanding of how to balance cationic density and hydrophobicity to design potent CAPs with low mammalian cytotoxicity. In our laboratory, there have been some efforts to design better WLBU peptides by replacing all the Val with Trp

residues in the hydrophobic face. Although a 24-mer with 11 Trp residues would be considerably more potent, it is highly likely that this peptide would display significant host toxicity. One way to overcome this problem is to create shorter WLBU peptides. Thus a 12- and 24-mers have already been synthesized, and their antibacterial efficacy will be soon evaluated. However, to completely explore this peptide series for improving the therapeutic window, the synthesis of more WLBU peptides differing by only 3 residues in length is warranted. For instance, we could create Arg-Trp peptides with 6, 9, 12, 15, 18, and 21 residues using the helical wheel analysis. In addition, some Trp residues could be replaced with Val in the most potent peptides to reduce mammalian toxicity, a reverse approach to that used in the initial design of the WLBU series. The smallest peptides (*e.g*, 6, 9 residues) could be made circular by introducing two Cys residues at the N- and C-termini or oligomeric by adding one Cys residue at either end of the peptide. The results of these studies could potentially lead to shorter peptides with similar potency to WLBU2 but with lower potential for host toxicity. It will take several years to design the WLBU peptides and evaluate their antimicrobial efficacy. However, because we have already developed the methodology, it should be less time consuming to examine selective toxicity, structural analysis, and *in vivo* efficacy.

5.5.2. Progress toward Clinical Applications

5.5.2.1. *Treatment of Bacteremia*

The convincing evidence for the potential application of WLBU2 to *P. aeruginosa infections* does not preclude further evaluation of the efficacy of this peptide in animal models. Because of biological differences between humans and mice, it is important to compare (in our mouse models) the efficacy of WLBU2 with that of standard antibiotics.

The polymyxins (*e.g.*, colistin) are the most common family of CAPs used clinically against Gram-negative infections, and tobramycin is a standard antipseudomonal agent. Some comparative studies between WLBU2, colistin, and tobramycin have already been initiated in Dr. Mietzner's laboratory (Omar Bakth) (137, 293, 305). WLBU2 (at much lower concentrations than colistin) demonstrated zones of bacterial growth inhibition comparable to those of colistin on agar plates against a tobramycin-resistant *P. aeruginosa* strain. It would be very informative to compare these three antimicrobial agents in our *in vivo* models against both tobramycin-sensitive and -resistant strains of *P. aeruginosa*. These studies could be concluded by comparative analysis of the pharmacokinetics of colistin and WLBU2. Such studies would provide fundamental information from which to predict the potential therapeutic effect of WLBU2 in a clinical setting.

5.5.2.2. *Treatment of Lung infections*

P. aeruginosa can colonize a variety of tissues, but it is most known for causing lung infections (pneumonia) (347). The application of WLBU2 to the treatment of lung infections could serve as an effective alternative therapy against MDR *P. aeruginosa*, particularly in CF and ventilator-associated pneumonia patients. An intratracheal (IT) infection model is currently being developed in collaboration with Dr. Jay Kolls (Children's Hospital, Pittsburgh, PA). In this model, both bacteria and peptide will be administered (in sequence) IT. Alternatively, the bacterial suspensions or the peptide (or both) could be given directly through the murine airway by nebulization. The method of aerosolization, although more technically complex, would be more reflective of a real clinical situation than the IT infection. A third approach would be to

deliver the peptide systemically. The advantage of the aerosolization and the systemic methods of administration is that they are both clinically applicable.

5.5.2.3. Application to Infections Associated with the Implantation of Medical Devices (Prophylaxis)

We have already demonstrated the *in vivo* prophylactic potential of WLBU2. In addition, other unpublished data from our laboratory have shown that resin-bound WLBU2 can rapidly sterilize a P. aeruginosa suspension in PBS (Omar Bakth). Our laboratory is planning to investigate the influence of WLBU2 on biofilm formation inside a catheter in vivo. The exploration of this avenue may lead to the prevention of infections associated with the implantation of medical devices.

In conclusion, although antimicrobial peptides must be developed in the context of specific applications, it is evident from the results of our current investigations that WLBU2 can be evaluated for several clinical applications. However, the significant changes in therapeutic effects due to small variations in peptide doses observed in our experimental models are unlikely to occur in humans. Therefore, it is imperative to use a larger range of peptide doses in future models to establish more accurately a therapeutic window for WLBU2.

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