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WLBU2 antimicrobial peptide as a potential therapeutic for treatment of resistant bacterial infections

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Summary
Antimicrobial resistance is considered as a major health problem, worldwide. It is significantly associated with high morbidity and mortality rates. The current antibiotics have limited therapeutic efficacy in providing treatment for multidrug resistant bacteria. Accordingly, research in the antimicrobial field has been directed toward the discovery of new agents to overcome bacterial resistance. Antimicrobial peptides have been extensively studied as potential antimicrobial agents with lower incidence of drug resistance in comparison to conventional antibiotics. WLBU2 is an engineered cationic antimicrobial peptide with promising antibacterial activity. It is composed of 24 amino acids including; 13 arginine, 8 valine and 3 tryptophan residues. Findings from *in vitro* and *in vivo* studies showed that WLBU2 is a potent peptide with broad spectrum activity against Gram-positive, Gram-negative, multidrug resistant and biofilm forming bacteria. In addition, WLBU2 appears as a salt resistant peptide with potential application for treatment of infections at conditions with disturbed normal salt homeostasis. Further, WLBU2 was found as antimicrobial peptide with limited host toxicity. Recent investigations have shown that combination of WLBU2 with conventional antibiotics can result in synergism against resistant bacteria. In this review we highlight the evidence supporting the promising properties of WLBU2 as an antibacterial agent with potential applications for treatment of infections caused by resistant bacteria.
Keywords: antimicrobial peptide, WLBU2, resistant bacteria, salt resistant, synergism, combination, non-cytotoxic

Introduction

Antimicrobial resistance (AMR) is recognized as a major global threat with negative impact on the public health systems around the world. [1] AMR has reached an alarming level since it is significantly associated with high morbidity and mortality rates. [2] According to the 2019 Antibiotic Resistance Threats report published by the United States Centre for Disease

Control and Prevention (CDC), more than 2.8 million antibiotic-resistant infections occur in the U.S. each year, and more than 35,000 people die. [3] Indeed, the World Health Organization (WHO) and the United Nations (UN) Interagency Coordination Group (IACG) on Antimicrobial Resistance have described the global impact of AMR as being very critical causing more than 700.000 deaths around the world, with expectations to reach 10 million at the end of 2050. [4] AMR is also well recognized to be associated with increasing health care cost. [5] This is mainly due to the need for expensive antibiotics as well as prolonged hospitalization and isolation of affected patients. [2,6]

Among Gram-positive pathogens, resistant *Staphylococcus aureus* has been described as the biggest threat. [7] Methicillin-Resistant *Staphylococcus aureus* (MRSA), known as being resistant to penicillin-like beta-lactam antibiotics, was first emerged 6 decades ago. [8] After that, MRSA infections have rapidly spread around the world, with high incidence were reported in many countries in Europe, USA, UK and the Asia-Pacific region. [9] Previous studies reported that most MRSA strains were inhibited by Vancomycin at MIC values of 0.125 to 1 μg/ml. [10] However, increased resistance of certain strains of *Staphylococcus aureus* toward Vancomycin have been observed. [11] This was first reported in Japan in 1996, with the discovery of clinical isolate with reduced susceptibility to Vanocmycin (MIC = 8 μg/mL), and was called Vancomycin intermediate *Staphylococcus aureus* (VISA). [12] In 2002, the U.S. reported the first *Staphylococcus aureus* isolate with complete resistance to Vancomycin (MIC ≥ 32 μg/mL) and was called Vancomycin-resistant *Staphylococcus aureus* (VRSA). [13]

Currently, substantial evidence points to the negative impact of Gram-negative pathogens, which are considered very common in the community. [3,14] Indeed, many of these were reported as being Multidrug-Resistant (MDR) including extended spectrum beta-lactamase-producing (ESBL) *Escherichia coli, Neisseria gonorrhoeae* and *Enterobacter cloacae,* [3,14-16] which are resistant to third generation Cephalosporins (Ceftriaxone and Ceftazidime) and Monobactams. [17]

Recognizing the fact that the current antibiotics have limited therapeutic efficacy in providing treatment for MDR Gram-positive and negative bacteria, new strategies have been developed in order to overcome antibiotic bacterial resistance. [6] Antimicrobial peptides (AMPs) have been extensively studied as potential antimicrobial agents with lower incidence of drug resistance in comparison to conventional antibiotics. [18-20] In this review we highlight the evidence of antibacterial activity of WLBU2 peptide as a novel engineered cationic antimicrobial agent with potential applications for treatment of infections caused by resistant bacteria.

Antimicrobial peptides

AMPs are a large family of low molecular weight peptides that have a key role in innate immunity. Most AMPs are cationic peptides and have the ability to kill and/or inhibit bacterial growth. [21] The exact mechanism of AMPs antibacterial effect is not completely understood. However, AMPs have been suggested to bind the bacterial cell membrane and cause disruption of the lipids components. [22] This is usually induced by electrostatic interactions between positively charged amino acids within the peptide and negatively charged lipids in bacterial cell membrane. [23,24] As a consequence, increasing the plasma membrane permeability and disruption of the plasma membrane will cause leakage of ions and metabolites as well as cessation of membrane-coupled respiration and biosynthesis that can contribute to bacterial cell death. [23,24]

WLBU2 antibacterial activity

WLBU2 is an engineered cationic peptide that contains 24 amino acids including; 13 arginine. 8 valine and 3 tryptophan residues in the hydrophobic face separated from each other by at least 7 amino acids (**Figure 1**). [25] Results from *in vitro* and *in vivo* investigations

revealed the potency of WLBU2 with broad spectrum activity against different types of microorganisms including bacteria and diverse *Candida* species. [26-32] Preclinical studies on *Pseudomonas aeruginosa* described WLBU2 as salt resistant peptide with potent inhibitory effects against bacterial growth and biofilm formation. [26,33,34] Further investigations on lung infections caused by *Pseudomonas aeruginosa* supported the antibacterial effects of WLBU2 as well as induction of protective proinflammatory responses upon treatment. [33,35] The antibacterial potency of WLBU2 was also reported against three oral microorganisms (*Streptococcus gordonii, Fusobacterium nucleatum*, and *Porphyromonas gingivalis*). [27] In addition, WLBU2 was found to have bactericidal activity against three highly pathogenic bacteria: *Francisella tularensis, Yersinia pestis* and *Burkholderia pseudomallei.* [28] Recently, WLBU2 has been shown to eliminate pneumonia and MRSA superinfection during influenza as well as antibiotic resistant surgical implant biofilms caused by *Staphylococcus aureus* and MRSA. [29,36] In addition, it has been found to be very effective in preventing ESKAPE (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii*, *Pseudomonas aeruginosa, Enterobacter cloacae*) pathogens' biofilm formation and attachment .[30] Recent results have revealed potent antibacterial effects against biofilms of MDR *Acinetobacter baumannii* and *Klebsiella pneumonia.* [31]

The values of minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) of WLBU2 from previous investigations showed that WLBU2 has fast killing effects on bacterial cells with MIC values ≤ l0 μM. [19,31,37] The MIC values of WLBU2 from previous reports were as 1.5–3.2 μM for extensively drug-resistant (XDR) *Acinetobacter baumannii*, 2.9–4.7 μM for XDR *Klebsiella pneumoniae*, and 9.3 μM for *Klebsiella pneumoniae* KP2 strain. [19] In addition, Deslouches et al have shown that the MIC values of WLBU2 were ≤ l0 μM against both Gram-negative and Gram-positive bacteria including MRSA, Vancomycin-resistant *enterococci*, *Klebsiella pneumoniae, Enterobacter aerogenes, Enterobacter cloacae, Escherichia coli, Pseudomonas aeruginosa, and Acinetobacter baumannii*.[37] Recent investigations found that the MIC was 7.943 μM for *Klebsiella pneumoniae* and 7.484 μM for *Acinetobacter baumannii* clinical isolates. [31] Of note, the MBC values for WLBU2 against previous bacterial isolates were found to be identical to the MIC values of respective bacteria, indicating that WLBU2 is bactericidal. [38]

The exact mechanisms for the antimicrobial effect of WLBU2 are yet to be elucidated. However, it was suggested to be mediated by electrostatic interactions between peptide' cationic amino acid residues and the negatively charged lipid molecules on the surface of bacterial targets. [26] The potent activity of WLBU2 may be attributed to the high cationic charge and the increased length of amino acids of WLBU2 (24 residues). [26] Previous studies reported that the antibacterial activity of cationic AMP might also be mediated by binding to bacterial DNA. [39] However, limited investigations have been conducted in this regard for WLBU2. Only recent findings have revealed that WLBU2 at up to the tested 200 μM was not able to delay DNA mobility. [31]

WLBU2 and host cell toxicity

WLBU2 has been considered as potent antimicrobial peptide without significant host cell toxicity. [31] A previous study showed that WLBU2 had no cytotoxic effect at concentration of ≤20 μM against peripheral blood mononuclear cells, upon testing with the hemolytic assay and MTT assay. [19] A recent study has also revealed that WLBU2 is not cytotoxic when evaluated against human skin fibroblasts. [31] The selective toxicity of WLBU2 against bacteria and low toxicity against host cells suggest that WLBU2 form weak interactions with eukaryotic membranes which are known to be highly rich with cholesterol. In comparison, WLBU2 might form robust electrostatic interactions with the negatively charged bacterial membranes. [40]

WLBU2 as salt resistant peptide

One of the major drawbacks with the use of AMPs, mainly the natural AMPs, is their limited antibacterial activity due to inactivation by physiological concentrations of salts including sodium chloride and divalent cations. [39] It has been shown that the antibacterial activity of well-studied natural AMPs (such as LL-37, human β-defensin-1, gramicidins, bactenecins, and magainins) was substantially reduced under salt conditions. [41] Turner et al. showed that MIC values of LL-37 and human neutrophil peptide-1 (HNP-1) were significantly increased when NaCl was added. [42] In comparison, Mohamed et al described engineered AMP, RRIKA and RR, as salt-resistant since their antibacterial activity against MRSA was retained in the presence of physiological concentrations of NaCl and $MgCl_2$. [39] Recent studies also have shown that the synthetic AMPs such as D-RR4 and Hp1404 are salt resistant with retained activity against Gram-negative bacteria including *Pseudomonas aeruginosa* and *Acinetobacter baumannii.* [43,44] WLBU2 appeared as salt resistant since it retained the antibacterial activity when tested in different concentrations of NaCl, $CaCl_2$ and $MgCl_2$ against Gram-negative *Pseudomonas aeruginosa* or Gram-positive MRSA strains. [26] This is considered highly important for treatment of infections in conditions with disturbed normal salt homeostasis. [26] The ability of WLBU2 to resist the effects of salts suggests that the chemical structure of WLBU2 has been well designed to relatively retain antimicrobial activity in the presence of NaCl and divalent cations concentrations that is considered a major challenge for natural peptides. In addition, it provides a selective advantage as potential therapeutics in physiological solutions. However, further studies are needed to provide more evidence for WLBU2 as a salt resistant peptide since salt sensitivity might be sometimes dependent on the test organism. [26]

WLBU2 and synergism effects with conventional antibiotics

Recently, the effects of synthetic AMPs in combination with conventional antibiotics have been investigated against many Gram-negative and Gram-positive bacteria including MDR strains with biofilm formation ability. [45-48] Studies showed the efficacy of using synthetic peptides in combination with conventional antibiotics to augment the treatment of murine cutaneous abscesses caused by difficult to treat pathogens including all ESKAPE and *Escherichia coli.* [49] Gopal et al also reported that synergism was obtained upon combination of conventional antibiotics (Cefotaxime, Ciprofloxacin, or Erythromycin) with four cationic antimicrobial peptides (HPME, HPMA, CAME and CA) against 19 MDR *Acinetobacter baumannii* isolates. [50] In addition, synergistic effects were observed upon combination of the antimicrobial peptide DP7 and antibiotics (Gentamicin, Vancomycin, Azithromycin, and Amoxicillin) against several MDR bacterial strains including *Staphylococcus aureus, Pseudomonas aeruginosa, Acinetobacter baumannii*, and *Escherichia coli.* [51] SPR741 peptide also potentiated the effect of conventional antibiotics against *Escherichia coli, Klebsiella pneumoniae*, and *Acinetobacter baumannii.* [52] Recent findings from combination treatment have shown that T3 and T4 antimicrobial peptides resulted in potentiation of Ampicillin and Oxacillin against MRSA clinical isolates. [48] A synthetic cationic peptide, Pexiganan, is currently in phase 3 clinical trials as a contemporary antimicrobial agent for the treatment of *Escherichia coli, Klebsiella pneumoniae, Citrobacter koseri, Enterobacter cloacae, Acinetobacter* species, and *Pseudomonas aeruginosa* infections associated with diabetic foot ulcers. [53]

Upon reviewing the literature, limited evidence is available regarding the combination of WLBU2 and other conventional antibiotics. Only a recent study has revealed that combination of sub-inhibitory concentrations of WLBU2 with Amoxicillin-Clavulanate or Ciprofloxacin for *Klebsiella pneumoniae*, and with Tobramycin or Imipenem for *Acinetobacter baumannii,* resulted in synergism with significant reduction in MIC values for some investigated isolates and ATCC strains. [31] Synergism and potentiation upon combination of WLBU2 and conventional antibiotics might be a result of increased

membrane permeability caused by the action of WLBU2 cationic peptide, which enhanced the penetration of antibiotics toward bacterial cells and thus, improved the drug efficacy and killing effects. [45,54] Further, it might result in obtaining synergistic effects and thus enhances efficacy. [54] Advantages of combination treatment may also include broadening the spectrum of antimicrobial coverage and reducing the needed doses of each antimicrobial agent and thus, drug toxicity. [54] However, future studies are needed to explore the potential of using WLBU2 in combination with conventional antibiotics against other MDR and biofilm forming bacteria.

Conclusion

Cationic antimicrobial peptides are emerging as potential antimicrobial agents that can be used as alternative or complement to conventional antibiotics in order to overcome drug-resistant infections. WLBU2 appears as a novel peptide with promising properties as summarized in (**Figure 2**). *In vitro* and *in vivo* studies showed that WLBU2 is potent antimicrobial agent with bactericidal effects and broad spectrum activity against many Gram-positive, Gram-negative, multidrug resistant and biofilm forming bacteria. The therapeutic potential of AMPs can be affected by physiological conditions including the presence of salts and divalent cations at infection site or body fluids. WLBU2 showed the ability to resist the effects of salts which provides a selective advantage as potential therapeutics in physiological solutions and suggests that WLBU2 has been successfully synthetized to resist different salts types and concentrations, that is considered a major drawback of natural AMPs. WLBU2 also appears as AMP with minimal cytotoxic effects against host cells. Recent studies have provided proof of concept that WLBU2 as a cationic antimicrobial peptide can be used in combination with conventional antibiotics to reduce antimicrobial drug resistance which is considered a major issue to any health care system.

Future studies on the ability of WLBU2, either alone or in combination with conventional antibiotics, to eradicate or prevent biofilm formation will increase our knowledge regarding WLBU2 antibacterial activity. This might be considered under normal conditions and in the presence of salts, serum and proteases. Limited evidence is available regarding the potential synergism between WLBU2 and other cationic AMPs and therefore, it might be considered for future studies. In addition, *in vivo* animal studies evaluating WLBU2 antibacterial activity as potential peptide for treatment of bacterial infections might be highly valuable to be carried in the future. Similar investigations could be conducted for many MDR Gram-positive and Gram-negative bacteria in order to characterize the spectrum of WLBU2 activity which is expected to be broad spectrum.

Investigations regarding the exact underlying mechanisms of the antibacterial activity of WLBU2 alone and combined with conventional antibiotics might be considered as future experiments, since the underlying mechanisms are still to be elucidated. These investigations might involve studies on morphological changes or effects at the molecular level including genes playing role in bacterial resistance or bacterial metabolism as well as other energetics aspects.

Competing interests

The authors declare no conflict or competing of interests.

Authors' contributions

First author: Writing - Original Draft, Writing - Review & Editing, Figure 2 preparation, Second author: Writing - Review & Editing. Third author: Writing - Review & Editing. All authors have critically reviewed and approved the final draft and are responsible for the content and similarity index of the manuscript.

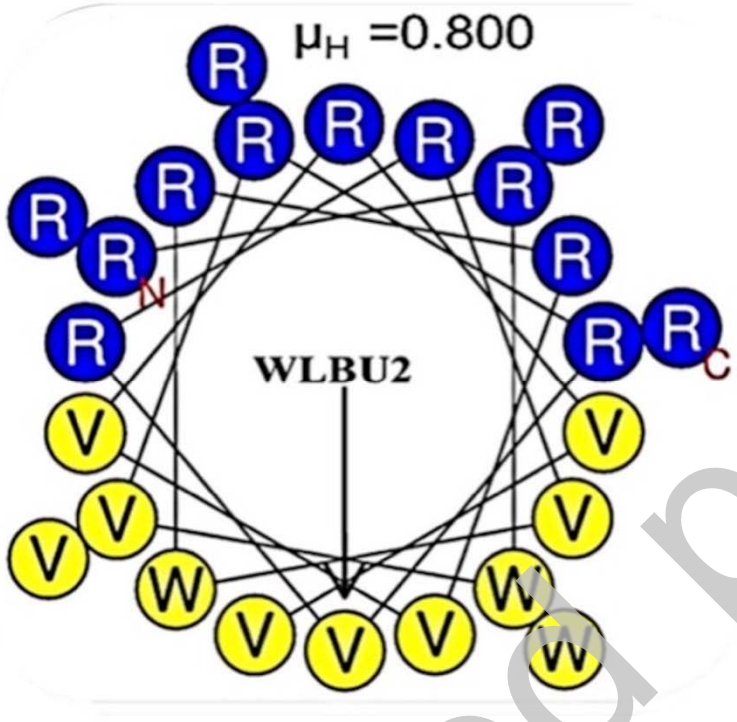

Figure 1. WLBU2 peptide structure. WLBU2 contains 24 amino acids including; 13 arginine (R). 8 valine (V) and 3 tryptophan (W) residues in the hydrophobic face separated from each other by at least 7 amino acids Adopted from Deslouches et al 2015,[37] license number 4927790008448.

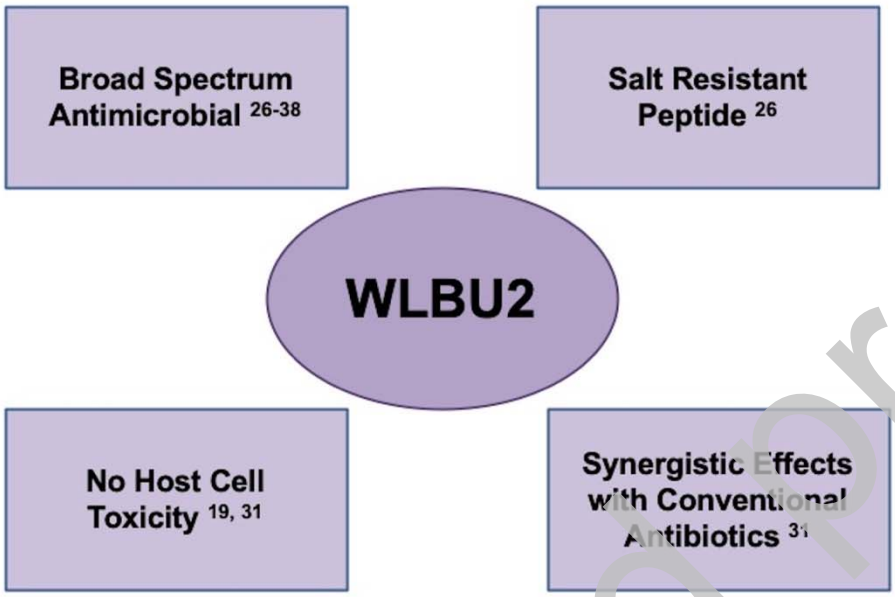

Figure 2. Properties of WLBU2 antimicrobial peptide.

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