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SYNERGISTIC EFFECT OF THE WLBU2 ANTIMICROBIAL PEPTIDE IN COMBINATION WITH COMMON ANTIBIOTICS AGAINST CLINICAL ISOLATES OF MULTI-DRUG RESISTANCE PATHOGENIC BACTERIA

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ABSTRACT

Antimicrobial resistance (AMR) is a global burden that negatively affect health care system and economy. Resistance pattern to traditional antibiotics increasing dramatically making these agents ineffective to treat infections caused by resistance pathogens. Recently, researches and studies are directed toward new therapeutic options including development of engineered cationic antimicrobial peptides such as WLBU2. In our study, Ceftriaxone, Ciprofloxacin and Ceftazidime in combined with WLBU2 were utilized against fifty clinical isolates of *Proteus mirabilis* (n=25) and *Pseudomonas aeruginosa* (n=25) to evaluate the activity and the effect of combination. The obtained results revealed that synergistic and additive effect were observed and achieved with significant ratio at low MIC and provide potent inhibitory and bactericidal effects.

KEYWORDS: Antimicrobial Peptides (AMPs), Engineered cationic peptides WLBU2, Multidrug resistance, *Proteus mirabilis*, *Pseudomonas aeruginosa*, Synergistic effect.

I. INTRODUCTION

Globally, Antimicrobial Resistance (AMR) is an emerging serious threat to public healthcare system. Increasing the cost of hospitalization and treatment of infectious diseases which negatively affect the economic situation for both developing and developed countries. The total cost of AMR has been complicated to calculate. However, the Centres for Disease Control and Prevention in the United States of America estimated the cost of AMR in healthcare field about 20 billion dollar, with extra cost affecting community with loss of productivity around 35 billion dollar.[1]

Significantly, morbidity and mortality have been increased dramatically regarding the wide spread of AMR across countries. World Health Organization (WHO) describe the current situation of AMR as a critical threat making traditional antibiotics ineffective to treat infectious diseases leading to increased risk of disease spread, sever illness, complicated treatment options and deaths. WHO estimated that around 700.000 people death every year because of AMR and expected that number may increase significantly to reach around 10 million at the end of 2050.[2] In addition to that, WHO launched the Global Antimicrobial Resistance Surveillance System (GLASS) to support the researches in order to collect, analyze and share data of AMR at international level.[3]

Recently, treatment of infectious diseases is increasingly complicated due the fact that AMR is a continuous phenomenon enabling bacteria pathogens to develop different type of resistance mechanisms against conventional antibiotics. During the last decades, many researches and studies were conducted to investigate and describe the mechanism of resistance. Significantly, genetic mutations play a major role in developing different mechanisms of resistance. Bacteria pathogens have been reported to resist antibiotics through alternation of drug target sites, change permeability of cell wall, pump out antibiotics agents by efflux pump and produce certain type of enzymes to deactivate antibiotics molecules.[4] Typically, bacterial enzymes are playing an essential role antibiotics resistance. For example, resistance to antibiotics resulted of production of deactivation enzymes is described by mutation process that enable activation of nitrofurantoin. Genetic mutation in nitroreductase gens are responsible to nitrofurantoin resistance and mainly associated with nfsA and nfsB genes. Also, ribE gene encodes special type of enzyme known as lumazine which works as a co-factor in biosynthesis of riboflavin.[5] In addition to that, Penicillin binding proteins (PBP) is a major component in

synthesis of peptidoglycan, which is the main component of bacterial cell wall. β-lactam resistance is produced by modification or replacement of bacterial Penicillin-Binding Proteins (PBP) by encoding mosaic PBP genes which is commonly reported as a resistance mechanism in *Staphylococcus aureus* pathogen.[6] In parallel to that, rapid spread of resistance to antibiotics based on aminoglycosides is mainly related to enzymatic modification produced by several types of enzymes known as aminoglycoside-modifying enzymes (AMEs). Those enzymes are encoded by certain genes localized in mobile genetic elements and represented by several isoenzymes with unique substrate that are modifying aminoglycosides' receptors at different position.[7] Moreover, efflux pumps are considered as an energy dependent transport protein founded in both gram-negative and gram-positive bacteria and located on the cytoplasmic membrane enabling bacteria to pump out antibiotics' molecules. Pump mechanism may be specific for one antibiotic molecule or multiple molecules which is responsible to develop multidrug resistance.[8]

Both gram-positive and gram-negative bacteria develop resistance to antibiotics agents. Regarding gram-positive pathogens, in 1960 *Staphylococcus aureus* was reported to develop resistance against second generation of beta-lactam antibiotics by modifying Penicillin-binding protein, during less than one year after the introduction of semi-synthetic Penicillin related antibiotics. Also, *Staphylococcus aureus* continuing to develop resistance to next generations of antibiotics including cephalosporins, and carbapenems. MRSA represent one of the most common multidrug resistance pathogens and have widely spread and cause severe infections.[9] On the other hand, gram-negative bacteria are adaptive pathogens developing resistance through different mechanisms and showed an increasing prevalence of resistivity. Most common resistance pathogens of gram-negative bacteria including; *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa.* Different types of infections are associated with gram-negative bacteria. Usually, *Pseudomonas aeruginosa and Proteus mirabilis* causing urinary trac infection, wound infection, skin and soft tissue infections, blood stream infections, gastrointestinal infection and pneumonia especially for hospitalized patients.[10] Resistance pattern among gram-negative bacteria showed that resistance to antibiotics acquired commonly by production of β-lactamases enzymes that hydrolyze β-lactam ring. Classification of β-lactamases resistance is based on molecular structure (Ambler classification) or functional similarities (Bush-Jacoby-Medeiros classification). *Proteus mirabilis* and *Pseudomonas aeruginosa* were reported to resist β-lactams antibiotics according to Ambler class C and D. Cephalosporinases and Oxacillinases were observed among certain type of enzymes including AmpC, OXA-23, OXA-24, OXA-58 cluster, and OXA-51/69.[11] In addition to that, other types of mechanisms were reported among gram-negative bacteria including efflux pump and modification of target site which is leading to an emerging threat of resistance pattern to most available antibiotics.[12]

Regarding to the multiple resistance mechanisms of gram-negative bacteria, treatment options among available conventional antibiotics are limited. However, carbapenems consider as first line antibiotics to treat infections caused by gram negative bacteria.[13] Pitout et al., 2015 declared that resistance to carbapenems has been an emerging challenge to public health.[14] One of the last restore antibiotic against gram-negative bacteria is glycylcycline, which is usually used to treat complicated skin and soft tissue infections.[15] Unfortunately, resistance to glycylcycline was reported early after few years of its approval to use in clinical applications.[16,17] Newer treatment options by combination of two or more antibiotic molecules has been performed to identify the necessary exposure needed to achieve full inhibition against multidrug resistance pathogens.[18,19]

Currently, new strategies have been developed to overcome the limitation of conventional antibiotics to treat multidrug resistance infections. Many researches and studies have been conducted to investigate the ability of Antimicrobial peptides (AMPs) to treat infections caused by multidrug resistance pathogens.[20,21] AMPs are an essential component of innate immune system working as first line defense, composed of heterogenous group of small molecules with less than one hundred amino acids.[22] Particularly, most AMPs are positively charged (cationic) with amphiphilic (hydrophilic and hydrophobic) properties among α-helical peptide molecules. Cationic properties play a significant role in binding with negatively charged bacterial cell membrane. Promoting electromechanical potential around bacteria cell membrane and inducing cell membrane damage which increase the permeability for larger molecules to enter bacteria cell leading to pathogens inhibition and death.[23] Significantly, engineered cationic amphipathic peptide WLBU2 is composed of 24 amino acid sequence, including 13 arginine. 8 valine and 3 tryptophan residues at the hydrophobic side separated by at least 7 amino acid leading to make WLB2 active in salt physiological concentration. Regarding to Deslouches et al. 2005 WLBU2 showed high antibacterial susceptibility to inhabit gram-negative bacteria including *Pseudomonas aeruginosa*.[24] In addition to that, WLBU2 was investigated against pulmonary infections caused by *Pseudomonas aeruginosa*, the obtained results demonstrated high potential of WLBU2 to treat respiratory infections.[25] Also, WLBU2 showed a promising potency to prevent biofilm formation with lower minimum therapeutic dose.[26] Significantly, AMPs showed preferential interaction and selective toxicity for microbial target which make AMPs as a promising future therapeutic with broad spectrum of antimicrobial activity.[27] Our study aims to investigate the potential of combination between traditional antibiotics with

WLBU2 against multidrug pathogenic bacteria; *Proteus mirabilis* and *Pseudomonas aeruginosa*.

II. METHODOLOGY

Fifty clinical isolates of *Proteus mirabilis* (n=25) and *Pseudomonas aeruginosa* (n=25) were isolated and stored at -80C° in microbiology labs at Royal Medical Services Hospitals. Conventional antibiotics; Ceftriaxone, Ciprofloxacin and Ceftazidime were chosen according to according to CLSI guidelines (2017) and utilized in combination with WLBU2 to perform susceptibility tests. Broth microdilution was used to evaluate minimum inhibitory concentration (MIC) of WLBU2 and traditional antibiotics. MICs of conventional antibiotic in combination with WLBU2 were determined by checkerboard assay to determine the fractional inhibitory concentration indices (FICIs) according to the following equation: FICI= MIC (antibiotic in combination) \ MIC (antibiotic alone). Synergistic effect was interrupted according to FICI value as the following: (FICI< 0.5: synergistic), (0.5<FICI<1: additive), (1≤FICI<4 indifference), and (FICI≥4: antagonism).

III. RESULTS

The results of conventional antibiotic, WLBU2 alone and in combination are summarized in the below table against *Proteus mirabilis* and *Pseudomonas aeruginosa*.

Table 1: MIC of WBLU2 alone and in combination with traditional antibiotics against *Proteus mirabilis* and *Pseudomonas aeruginosa.*

Pathogens MIC	***Proteus mirabilis* (n=25)**	***Pseudomonas aeruginosa* (n=25)**
Ceftriaxone	64 µg/mL	32 µg/mL
Ciprofloxacin	64 µg/mL	32 µg/mL
Ceftazidime	128 µg/mL	64 µg/mL
WLBU2 alone	1.56 µg/mL, (n = 3)	1.56 µg/mL, (n = 2)
	3.12 µg/mL, (n = 16)	3.12 µg/mL, (n = 17)
	6.25 µg/mL, (n = 4)	6.25 µg/mL, (n = 5)
	12.5 µg/mL, (n = 1)	12.5 µg/mL, (n = 1)
WLBU2 in combination Ceftazidime	32 µg/mL, (n = 6)	16 µg/mL, (n = 5)
	64 µg/mL, (n = 15)	32 µg/mL, (n = 16)
	128 µg/mL, (n = 4)	64 µg/mL, (n = 4)
WLBU2 in combination Ceftriaxone	16 µg/mL, (n = 7)	8 µg/mL, (n = 6)
	32 µg/mL, (n = 14)	16 µg/mL, (n = 15)
	64 µg/mL, (n = 4)	32 µg/mL, (n = 4)
WLBU2 in combination Ciprofloxacin	16 µg/mL, (n = 8)	8 µg/mL, (n = 7)
	32 µg/mL, (n = 15)	16 µg/mL, (n = 17)
	64 µg/mL, (n = 2)	32 µg/mL, (n = 1)

MIC values showed that *Proteus mirabilis* was resistance to all examined conventional antibiotic (Ceftriaxone MIC=64 µg/mL), (Ciprofloxacin MIC=64 µg/mL) and (Ceftazidime MIC=128 µg/mL). As same as, *Pseudomonas aeruginosa* was resistance to (Ceftriaxone MIC=32 µg/mL), (Ciprofloxacin MIC=32 µg/mL) and (Ceftazidime MIC=64 µg/mL).

While the results of WLBU2 against clinical isolates showing that MIC and MBC value were similar and ranged from 1.5 to 12.5 µg/mL. Significantly, combination of traditional antibiotics with WLBU2 achieve different type of effect including synergistic and additive effects.

IV. DISCUSSION

Our results revealed that synergistic effect can be achieved by using WLBU2 in combination with conventional antibiotics including Ceftriaxone, Ciprofloxacin and Ceftazidime.

Figure 1 summarized the obtained results of combination against *Proteus mirabilis* isolates, synergistic effect was achieved by combination of WLBU2 with Ceftazidime at MIC = 32 µg/mL, for (n = 6/25) isolates. While additive effect was observed among (n = 15/25) at MIC = 64 µg/mL. Also, indifferent effect was reported through (n= 4/25) at MIC 128 µg/mL. The achieved ratio of synergistic effect was around 25% compared to Additive ratio which is about 60%.

As well as, combination of Ceftriaxone with WLBU2 showed that synergistic effect was reported through

(n=7/25) at MIC 16 µg/mL compared to additive effect which was observed at MIC = 32 µg/mL through (n=14/25). The obtained ratio of synergistic and additive effect was (28%) and (56%) respectively.

In addition to that, combination of Ciprofloxacin with WLBU2 showed significant ratio of synergistic effect with ratio around (32%) at MIC 16 µg/mL through (n=8/25). Additive effect was reported at MIC = 32 µg/mL for (n=15/25) which represents a ratio of (60%).

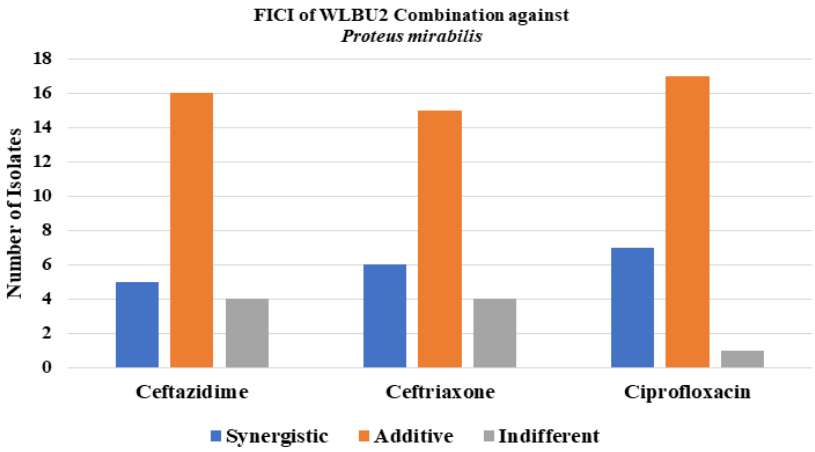

Figure 1: FICI of WLBU2 combination against *P. Mirabilis.*

In parallel to that, figure 2 describes the obtained results of combination between traditional antibiotics and WLBU2 against *Pseudomonas aeruginosa*, showing that synergistic effect was achieved among combination of Ceftriaxone, Ciprofloxacin and Ceftazidime at MIC 8 µg/mL (n=7/25), 8 (n=6/25) µg/mL and 16 µg/mL (n=5/25) respectively. The obtained ratio of synergistic effect ranged from (25%) to (32%). Also, additive effect was observed among (n=16/25) for Ceftazidime at MIC = 32 µg/mL, and among (n=15/25) and (n=17/25) for Ceftriaxone and Ciprofloxacin at MIC 16 µg/mL. while indifferent effect was reported at (n=1/25) for Ciprofloxacin, and (n=4/25) for Ceftazidime and Ceftriaxone.

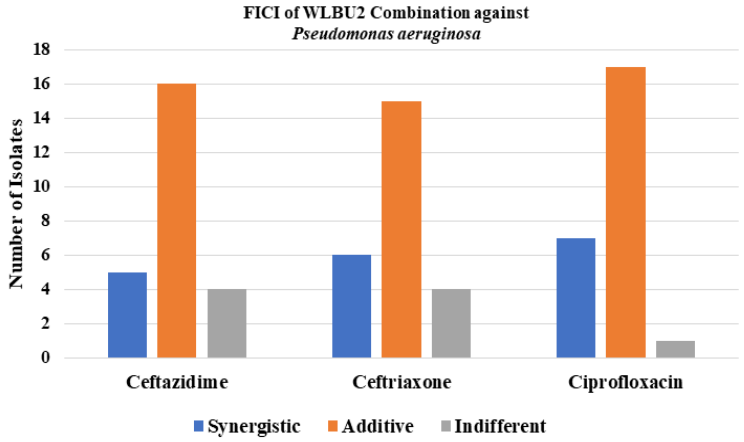

Figure 2: FICI of WLBU2 combination against *Pseudomonas aeruginosa.*

Our finding utilized gram-negative isolates of multidrug resistance pathogens; the obtained results support that combination of WLBU2 with classical antibiotics is a promising treatment option for resistance pathogens. In addition to that, our findings are compatible with recent researches and studies which investigated the effect of combination of WLBU2. For example, Deslouches et al. 2005 investigated different sequences of antimicrobial peptides including; LBU, LL37 and WLBU2 against *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The obtained results demonstrated that AMPs can be designed and modify based on cationic characteristics of Arg and Val residues on the hydrophobic face. Also, the antimicrobial activity of AMPs can be improved by increasing the chain length, where as 24 residues of WLBU2 demonstrated optimal selectivity against examined pathogens.[24] Also, Sierra et al. 2017 declared that AMPs are a promising therapeutic option against different types of microorganisms including fugue, bacteria and even cancer cells. They found that

synergistic effect can be achieved by combination between AMPs and traditional antibiotics such as combination between colistin with glycopeptides which showed a synergistic effect *in-vitro*.[28]

Furthermore, Deslouches et al.2015 investigated the potency of WLBU2 and WR12 engineered cationic antimicrobial peptides against 142 isolates of the most common multidrug resistance pathogens. The results showed that utilization of WLBU2 and WR12 *in-vitro* significantly improve antimicrobial spectrum against multidrug pathogens and provide less toxic treatment options compared with traditional antibiotics.[29] Moreover, Jiang et al. 2019 examined the ability of AMPs (α-4 short) against *Pseudomonas aeruginosa* isolates. Finding that derived AMPs (α4-short) suppress the growth of multidrug resistance bacteria and prevent biofilm formation at low concentration 2 µM.[30] Finally, Kang et al. 2017 reviewed the clinical potential of AMPs as therapeutic option against infections caused by multidrug resistance. AMPs have a synergistic effect when co-administrated with conventional antimolecules and provide a great potential to treat infections caused by multidrug resistance pathogens.[31]

V. CONCLUSION

Antimicrobial Peptides (AMPs) showed a promising potential of antimicrobial agents that can be utilized in combination with conventional antibiotics or alone to overcome multidrug resistance, and suggested to provide an extended spectrum of activity to develop alternative therapeutic agents. In our study, combination of conventional antibiotics with cationic engineered antimicrobial peptides (WLBU2) showed that synergistic and additive effect can be achieved with significant ratio against clinical isolate of multidrug resistance pathogens (*Proteus mirabilis* and *Pseudomonas aeruginosa)*. Also, MIC and MBC value of WLBU2 were identical and ranged between (1.56 – 12.5) µg/mL indicating that low concentration provide inhibitory and bactericidal effects.

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