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Synergistic Effect of Antimicrobial Peptide LL-37 and Ciprofloxacin against Multidrug Resistant *Pseudomonas aeruginosa* Isolated from Burn Infections

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Abstract: *Pseudomonas aeruginosa* is one of the most prevalent opportunistic bacteria, with a high activities rate in patients with burns and weakened immune systems. This study aimed to determine the inhibitory activity of the antimicrobial peptide LL-37 in combination with the antibiotic Ciprofloxacin against multidrug resistant isolates of *Pseudomonas aeruginosa* isolated from patients with burn infections. In this study, the checkerboard broth microdilution method was used to examine the antibacterial properties of the combination of LL-37 and Ciprofloxacin to determine the fractional inhibitory concentrations (FICs) at the concentrations (7.8-0.060 µg/ml) for selected eight multidrug resistant isolates. The results of synergism revealed that the concentrations with inhibitory activity for antimicrobial peptide LL-37 and ciprofloxacin were reduced to very low concentrations with FIC ranging from (0.0024-0.26 µg /ml). According to the synergistic effect, the MIC of LL-37 was reduced from 250 to 0.97 µg/ml for the isolate P1, while for the isolate P32 the MIC OF Ciprofloxacin was reduced from 1000 to 0.12 µg/ml. In conclusion, the current findings indicated that LL-37 and Ciprofloxacin combination was effective against multidrug-resistant *Pseudomonas aeruginosa* strains and suggested a new alternative option for burn infection treatment.

Keywords: Antimicrobial peptide, Burns, checkerboard, LL-37, *Pseudomonas aeruginosa*, synergism.

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Introduction

Pseudomonas aeruginosa (*P. aeruginosa*) is a Gram-negative opportunistic pathogen that infects patients leading to cystic fibrosis, burn wounds, immunodeficiency, chronic obstructive pulmonary disorder (COPD), cancer, and severe infection requiring ventilation, with increasing of its antibiotic resistance especially in strains from burn and wound infections(1). Due to an increase in bacterial resistance to current therapies as well as a decrease in the discovery of novel antibiotics, bacterial infections pose a rising threat to world health. As a result, there are more preventable illnesses and fatalities over the world (2). To effectively treat diseases that are resistant to medication, new antibacterial treatment must be found immediately. The need for such novel approaches has prompted researchers to investigate into naturally occurring antimicrobial agents that might be used to eliminate bacteria. The effective creation of new treatment approaches may benefit from the repurposing and modification of recognized natural antimicrobial proteins. Antibacterial peptides (AMPs) are prevalent and exhibit broad-spectrum antimicrobial

activity. They have received substantial research as a potential solution to deal with bacteria that are resistant to numerous drugs (3).

The single human cathelicidin peptide LL-37 has been shown to have antimicrobial and anti-biofilm activities against multiple Gram-positive and Gram-negative human pathogens, and have wound-healing effects on the host. The combination of the anti-biofilm effect and wound-healing properties of LL-37 may make it highly effective in resolving polymicrobially infected wounds when topically applied. Such a peptide or its derivatives could be a platform from which to develop new therapeutic strategies to treat biofilm-mediated infections of wounds (4). However, because bacteria can mutate quickly, new methods are needed to prevent bacterial resistance to AMPs from developing (5). In this research, go through how to employ AMP in combination with other antibacterial agent to create an antibacterial synergisim, where the combined antimicrobial effect is greater than the sum of the effect of the individual treatments. Antibacterial synergy may be able to slow the development of bacterial resistance. can cause cell death and damage. The barrel-stave, toroidal, and carpet classes of AMP-induced membrane pores have all been put forth (6). A potential way to reduce the risk of drug-resistance to AMPs in clinical settings is to use AMPs in conjunction with other antimicrobials, focusing on combinations that lead to effective antimicrobial synergies. Synergistic combinations that have multiple targets in independent pathways could require two independent and simultaneous sets of mutations to address both challenges (7). The aim of this study is to evaluate the role of the antimicrobial peptide LL-37 as inhibitory agent in combination with the antibiotic Ciprofloxacin against multidrug resistant isolates of *Pseudomonas aeruginosa* isolated from patients with burn infections.

Materials and methods

The antimicrobial peptide LL-37 (amino acid sequence: LLGDFFRKSKEKIGKEFKRIVQRIK DFLRNLVPRTES) was synthesized by the conventional solid-phase peptide synthesis protocol and were obtained from (Eurogentec, Belgium). The purity of LL-37 (molecular weight 4492) was 98.5% (as determined by high-performance liquid chromatography) was used in this study. Ciprofloxacin was used as a second-generation fluoroquinolone. Ciprofloxacin were procured from (MAST, UK), and is commonly prescribed to treat *P. aeruginosa* infections. Eight multidrug resistant isolates of *P. aeruginosa* from burn infections from Baghdad hospitals, and identified by VITEK2 system (bioMérieux, France).

Minimum inhibitory concentration

The broth microdilution method was used to determine the (MIC) of the LL_37 peptide and ciprofloxacin using the 96-well microtiter plate. The working solution of both were prepared at 4000 µg/ml in D.W to obtain final concentrations (1000-7.8 µg/ml).100µl of each prepared solution of peptide and antibiotic was introduced into the first wells in row A with 100µl broth in wells except positive and negative control. Rows B-H in columns had 100 µl of the broth alone. Two fold serial dilutions using micropipette were done systematically down the columns (from rows A-H).

Amount of 100 µl was removed from the starting concentrations in row

A and transferred to the next row with the 100µl broth, properly mixed, and the procedure was repeated up to the last row (H) where the last 100µl was discarded. This brings the final volume in all the test wells with the peptide to 100 µl except the column which had 200 µl of the broth that served as negative control. 100µl of bacterial inoculum was transferred into all the wells except the negative control.

Microtiter plates were incubated at 37 ºC for 18-20 hrs. After incubation, 20 µl of resazurin dye was added to all the wells and incubated for 30 minutes to observe any color changes. The Minimum Inhibitory Concentrations were determined visually in broth micro dilutions as the lowest concentrations of the peptide and antibiotic at which no color changed from blue to pink in the resazurin broth assay (8).

Fractional inhibition concentration (FIC)

The antibacterial activities of the combination of LL_37 and Ciprofloxacin were investigated using Checkerboard Broth Microdilution Method. Two-fold serial dilutions of LL_37 and Ciprofloxacin were prepared (working solution 1000 µg/ml) in the first experiment, and (62.5 µg/ml) in the second experiment to obtain final concentrations (125-0.97 µg/ml) and (7.8-0.060 µg/ml), respectively. 50µl aliquots of each component was placed into wells of sterile 96 well plate (third plate).

Already prepared bacterial inoculum (100 µl each) was added in respective wells and plates were incubated at 37ºC for 18-20h of incubation. After the incubation period, the plate was taken out and 20 µl of resazurin dye were added and the plate was taken back to the incubator for 20min.

Then the MIC was identified, and the FIC was calculated according to the formula:

$$FIC\ index = FIC\ A + FIC\ B = \frac{MIC_A^{Combination}}{MIC_A^{alone}} + \frac{MIC_B^{Combination}}{MIC_B^{alone}}$$

Where, A and B are the combined (in a single well) MICs of each antibiotic, and MICA and MICB are the individual MICs of each medication.

The FIC was determined as the wells without any visible growth. Synergy occurred at an FIC ≤ 0.5, antagonism at FIC > 4.0 and no interaction occurred at FICs > 0.5 to ≤ 4.0 (9).

Results and Discussion

The MICs for the combination of ciprofloxacin and LL-37 peptide against *P. aeruginosa* isolates. In order to find the MICs of the 2 inhibitory agents, The first experiment include using Ciprofloxacin and LL-37 peptide at concentrations with the range 125-0.97 µg/ml, and the results of the MICs of each agent and MICs of the combinations showed that the bacterial growth were inhibited at all concentrations used (Figure 1). Then when the concentration was lower, the ranged reached 7.8 0.060 µg /ml.

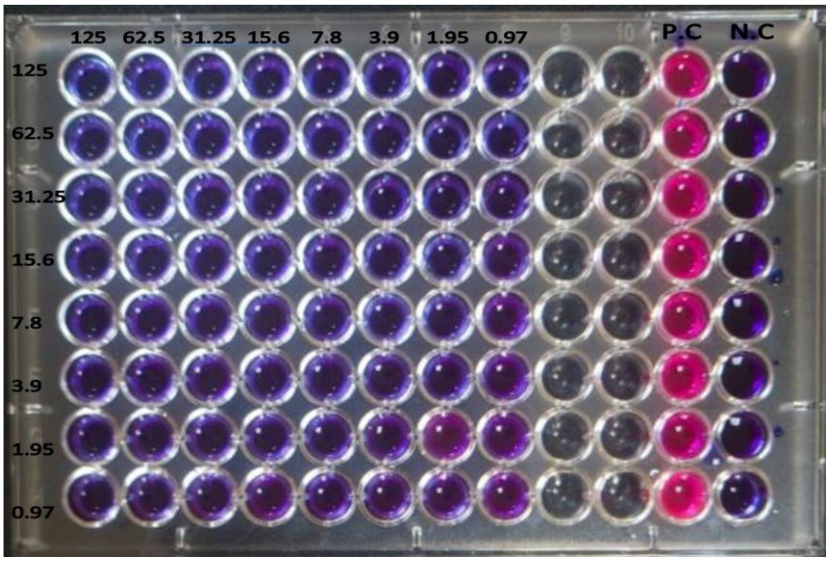

Figure (1): The minimum inhibition concentrations (MICs) of Ciprofloxacin and LL-37 peptide and for the combinations against *P. aeruginosa* isolate by checkerboard assay at concentrations (125 - 0.97 µg /ml).

The results of MICs of the concentrations ranged 7.8 0.060 µg /ml as showed in Figure (2) and Table (1), revealed that the concentrations with inhibitory activities for antimicrobial peptide LL-37 and ciprofloxacin were reduced to very low concentrations with FIC ranging from (0.0024-0.26 µg /ml). According to the synergistic effect, the MIC of LL-37 was reduced from 250 to 0.97 µg/ml for the isolate P1, while for the isolate P32 the MIC of Ciprofloxacin was reduced from 1000 to 0.12 µg/ml.

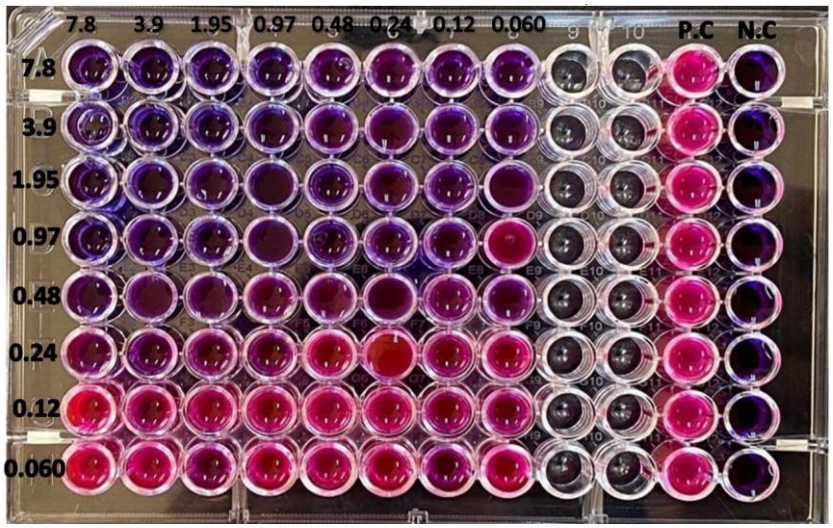

Figure (2): The Minimum inhibition concentrations (MICs) of ciprofloxacin and LL-37 peptide and for the combinations against *P. aeruginosa* isolate by checkerboard assay at concentrations (7.8-0.060 µg /ml).

The results of FIC Index showed that very low concentrations from the peptide LL-37 and the antibiotic ciprofloxacin exhibited significant inhibitory activates against the eight multidrug resistant isolates of *P. aeruginosa*.

Table (1): The Fractional inhibitory concentrations (FICs) of LL-37 peptide, ciprofloxacin and the combination of LL-37 peptide and ciprofloxacin by checkerboard method against *Pseudomonas aeruginosa* isolates.

Samples	MIC in combined for LL_37 peptide	MIC for LL_37 peptide	MIC in combined for ciprofloxacin	MIC for ciprofloxacin	FIC	FIC INDEX
P1	0.97	250	0.24	125	0.0058	Synergistic
P2	3.9	15.6	0.12	7.8	0.26	Synergistic
P13	0.97	15.6	0.24	250	0.06	Synergistic
P16	0.97	125	0.24	1000	0.008	Synergistic
P19	0.24	125	0.12	250	0.0024	Synergistic
P29	0.24	62.5	0.12	500	0.004	Synergistic
P30	3.9	125	0.12	250	0.03	Synergistic
P32	0.24	62.5	0.12	1000	0.003	Synergistic

FIC= Fractional Inhibitory Concentration, MIC = Minimum Inhibitory Concentration

The peptide LL-37 disrupted the integrity of the bacterial membrane. Also induced leakage of cell components, including nucleotides and even proteins (10). LL-37 affected biofilm formation of *P. aeruginosa* by decreasing the attachment of bacterial cells, stimulating twitching motility, and influencing two major quorum sensing systems (Las and Rhl), leading to the downregulation of genes essential for biofilm development (11).

The previous findings of Kampshoff *et al.* (12), demonstrated that the combination of melimine and ciprofloxacin showed synergistic activity against antibiotic sensitive or resistant strains of *P. aeruginosa* and with FIC values ≤0.5. Where, Combinations of AMPs and the fluoroquinolone ciprofloxacin is a promising method for reducing resistance to the fluoroquinolone of *P. aeruginosa*. The findings of Morroni *et al*. (13) revealed that LL-37 exhibited a good activity against carbapenemase-producing *E. coli* at the concentrations from 16 to 64 mg/l. Checkerboard assays demonstrated synergistic effect of LL-37 and colistin combination against all tested strains, further confirmed by time-kill and post antibiotic effect assays (13).

In order to increase the efficiency of the antibiotics and stop or delay the emergence of antibiotic resistance, combination therapies combining KR-12-a5 and its analogs with conventional antibiotics were assessed. Comparing our results to those of others, we can see that *P. auroginosa* performed better with lower doses of the LL-37 peptide and ciprofloxacin, with a FIC range of (0.0024-0.26 µg/ml). The using of AMP with an antibiotic could be an effective strategy. Synergistic antibacterial combinations with AMPs could enable bacterial pores to stay open for longer

durations, prevent pore repair, increase perturbation of bacterial intracellular functions, or convey other independent but complementary bacterial killing mechanisms. These mechanisms may potentially increase antimicrobial efficacy, decrease resistance, and reduce host toxicity (14). The hybridization and synergism strategy in developing AMPs as potential antimicrobial therapeutics with reduced toxicity profiles that could be efficiently employed to eradicate resistant bacterial strains and enhance the selectivity and toxicity profiles of native AMPs (15,16).

Conclusion

This study showed that the combination of the antimicrobial peptide LL-37 and ciprofloxacin worked synergistically against multidrug resistant *P. aeruginosa*.

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