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Evaluation of the antibacterial activity of human cathelicidin peptide-LL-37 in the presence of acidified nitrite

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Safaa Toma Hanna Aka *

Abstract

Background and objective: Bacterial resistance to conventional antibacterial agents has increased recently and this resistance results in complicated infections. Multiple protective mechanisms can evolve in mammalians to maintain the body protected from infections. Human cathelicidin antimicrobial peptide LL-37 and acidified nitrite are important components of the innate immune system that partake in preventing infections. Cathelicidin peptide LL-37 can be produced by epithelial tissues as well as by macrophages after microbial infections. This study was carried out to evaluate the antibacterial activity of LL-37 and acidified nitrite (AN) both individually and combined for their effect against the standard the strains of *E.coli* ATCC 25922 and *S. aureus* ATCC 25923.

Methods: Flat-bottom micro well plates (96 wells) were used for the determination of bacteriostatic activity. Sodium nitrite ($NaNO_2$) and ascorbic acid (AA) were used to produce acidified nitrite (AN). The singular and combined forms of the antibacterial agents were used for evaluating the antibacterial activities of LL-37 through estimation optical density values at 480nm (OD_{480nm}).

Results: The LL-37 peptide showed antibacterial activity against *E.coli* ATCC 25922 and *S. aureus* ATCC 25923. The antibacterial efficacy was enhanced when the peptide was tested in combination with AN (P <0.001). In contrast, the combination of LL-37 with NaNO2 and AA has an antagonistic effect (P <0.001) on its antimicrobial properties.

Conclusion: The combination of LL-37 with AN has a synergistic on the peptide's antimicrobial effect. Therefore, LL-37 which might show little antibacterial activity when used alone can provide protection when used in combination therapy with other antimicrobial agents.

Keywords: Antibacterial; Cathelicidin LL-37; Acidified nitrite.

Introduction

Many pathogenic bacteria combine their virulence factors for maximum effects in their infected hosts. This synergistic process leading to pathogenesis involves a number of events including adhesion and building biofilm, which ultimately results in complicated infections.[1] Many organisms can produce antimicrobial agents to defend against infection.[2] The same approach can be seen in mammalians to maintain organs and tissues protected from life-threatening microbial pathogens. Multiple protective mechanisms have evolved; one of which involves the production of antimicrobials peptides (AMPs).[3] AMPs can be polypeptides, proteins and /or lipids that vary in size, structure and all represent important protective parts of the innate immune system. These are involved in preventing establishing foci of infection and aid in bacterial clearance.[4] In human, cathelicidin antimicrobial peptide (hCAP-18/LL-37) is a type of AMPs secreted by epithelial tissues during infections. The peptide acts as a second line of defense for mucosal surfaces.[5] Cathelicidin LL-37 was originally discovered as produced by macrophages following their activation by bacteria, viruses and fungi. AMPs are typically expressed in phagocytic cells constitutively and are stored in cytoplasmic

* Department of Pharmacogonsy, College of Pharmacy, Hawler Medical University, Erbil, Iraq.

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granules, which are then released into developing phagolysosomes.[6] Dietary intakes of nitrate generate nitrite, which is acidified in the body leading to the generation of a number of reactive nitrogen species. Therefore, acidified nitrite (AN) has been considered to be another important host defense molecule with antimicrobial activities. Its effects overlap with the nitric oxide that is believed to be important in microbial killings.[7] *In vitro* studies of AN has shown it to possess antimicrobial properties against various types of bacteria.[8,9] The antimicrobial activities of reactive nitrogen species are exemplified by nitric oxide (NO).[10] The aim of the current study was to evaluate the antibacterial effects of human cathelicidin LL-37 *in vitro* used either alone or in combination with AN to find out if they have synergistic or antagonistic effects against standard strains of *E.coli* ATCC 25922 and *S. aureus* ATCC 25923 when used together. This idea is based on the knowledge that the two antibacterial agents can be present together at sites of infection.

Methods

Bacterial Inoculums

Standard *E. coli* ATCC 25922 and *S. au-reus* ATCC 25923 strains (provided by Bacteriology laboratory/Rizgary Teaching Hospital) were tested for the purpose of this investigation. All experiments used 18 hrs bacterial cultures. McFarland 0.5 tubes were adjusted so that the bacterial inoculums produced 10^5 CFU/ml.

Determination of antibacterial activities

Human cathelicidin peptide (hCAP/LL-37) was chemically synthesized (purchased from Agrisera - Sweden). All tests were carried out using disposable, flat-bottom micro well plates (96 wells). Sodium nitrite ($NaNO_2$) solutions were prepared at final concentrations of 5, 10, 20, 40, 80, 160, 320, 640, and 1,024μM in nutrient broth solution and added to the individual micro wells. Selected wells as per need were acidified by 5mM ascorbic acid (AA) to give a final pH 5.[11] To determine the antibacterial activity of LL-37, the experiments were repeated using final concentrations of 2, 4, 8, 16, 32, 64μg/ml of LL-37. Control wells contained bacterial growth medium only. Two separated micro well plates were separately tested for AN and LL-37. To each well of the first plate 100μL of AN was added while 100μL of LL-37 to all wells on the other plate. One hundred μL of the bacterial suspension was added to all wells as recommended by (Clinical Laboratory Standard Institute (CLSI). The plates were sealed and incubated for 24 hrs at 37C°. The inhibitory effects of the test materials on bacterial growth were determined by measurement of the OD_{480nm} of the wells using an ELISA plate reader (ELX800 Biotek / USA). The MIC was defined as the lowest concentration that no visible growth had taken place after 24 hrs.

Treatment with the combination of LL-37 and acidified nitrite (AN)

To determine the effect of acidified nitrite (AN) on the antimicrobial activity of LL-37, the experiments were repeated using the combination of solutions containing the two agents to give final concentrations of LL-37 ranging from 2-64μg/ml and fixed concentration of AN at (10μM NaNO2+5 mM AA) to achieve final pH 5 as reported by Carlsson et al.[10]

Statistical analysis

All experiments were carried out in triplicates. The mean ± SD were estimated for all test replicates. Independent-samples t test was used for data analysis. A *P* value of ≤0.05 considered statistically significant.

Results

The study assessed the bacteriostatic efficacy of the LL-37, $NaNO_2$, and AN. Results of MICs concentrations are summarized in Table 1.To assess the effectiveness of the antibacterial activity of the compounds, LL-37 was tested either as a single treatment or in combination with fixed concentrations of $NaNO_2$ at 10μM and 5mM of AA at pH 5. All treatments

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reduced the mean±SD of optical density at OD_{480nm} when cultured with *S.aureus* ATCC 25923 and *E.coli* ATCC 25922 in comparison with the controls as shown in (Figures 1 and 2). Results presented in Figures 1 and 2 provide evidence for the antibacterial efficacy of treatment with either LL-37 alone or the effect when used in combination with $NaNO_2$ in killing. The study assessed the compound for any synergistic /antagonistic interactions at pH 5 and determined whether the rate of killing by LL-37 increases or decreases when used in combination with $NaNO_2$. The results revealed antagonistic effect when LL-37 was used in combination with $NaNO_2$ or AA. LL-37, when used alone, had antibacterial activity greater than when combined with either $NaNO_2$ or AA.

Table 1: MICs of LL-37, $NaNO_2$, and AN against *E. coli* ATCC 25922 and *S.aureus*

Antibacterial agents	MICs	
	***E.coli* ATCC 25922**	***S.aureus* ATCC 25923**
LL-37	64 µg/ml	64 µg/ml
$NaNO_2$	>1,024 µM	>1,024 µM
AN	>320 µM	>320 µM

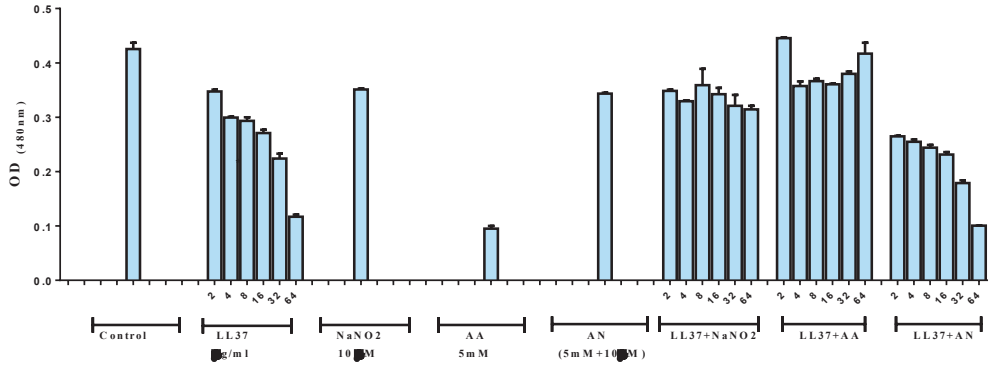

Figure 1: Mean±SD of OD_{480nm} values obtained for LL-37 with and without $NaNO_2$ and $NaNo_2$ alone as measures of their antimicrobial effects against *S.aureus* ATCC 25923.

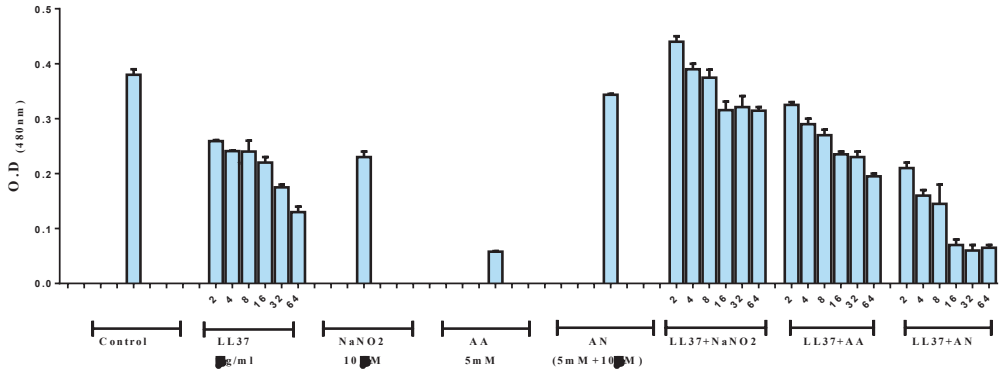

Figure 2: Mean±SD of OD_{480nm} values obtained as measures of the antibacterial effects of LL-37 with and without $NaNO_2$ and $NaNo_2$ alone against *E.coli* ATCC 25922.

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The data in Table 2 and Table 3 show that the mean±SD of the OD_{480nm} were significantly higher when in combined LL-37 was used in combination with $NaNO_2$ and LL-37 with AA than when LL-37 was used alone (*P* <0.001 and *P* <0.01), respectively. In contrast, the results show that the synergistic interaction between LL-37 and AN was more marked than the activity of either agent alone. Table 2 and Table 3 show the means ± SD decreases in OD_{480nm} with the combination of LL-37 and AN in comparison with the peptide LL-37 alone at the different concentrations tested against *S.aureus* ATCC 25923 and *E.coli* ATCC 25922 (*P* <0.05 and *P* <0.01, respectively).

Table 2: The mean± SD of OD_{480nm} resulting from the use of antimicrobial agents against *S.aureus* ATCC 25923.

LL-37 (µg/ml)	(Mean± SD) at OD_{480nm} of *S.aureus* ATCC 25923						
	LL-37	LL-37+NaNO2	*P* value*	LL-37+AA	*P* value•	LL-37+AN	*P* value†
2	0.346±0.005	0.333±0.005	0.047	0.443±0.005	0.001	0.253±0.01	0.001
4	0.293±0.005	0.320±0.010	0.024	0.350±0.010	0.003	0.246±0.005	0.001
8	0.286±0.115	0.353±0.030	0.49	0.363±0.056	0.002	0.233±0.005	0.006
16	0.263±0.005	0.336±0.011	0.002	0.353±0.057	<0.001	0.226±0.005	0.001
32	0.216±0.115	0.320±0.020	0.004	0.353±0.015	<0.001	0.173±0.005	0.011
64	0.106±0.057	0.310±0.010	<0.001	0.376±0.020	0.001	0.096±0.057	0.101

*** Comparing (LL-37+$NaNO_2$) with LL-37**
• Comparing (LL-37+AA) with LL-37
† Comparing (LL-37+AN) with LL-37

Table 3: The mean± SD of OD_{480nm} from using antimicrobial agents against *E.coli* ATCC 25922.

LL-37 (µg/ml)	OD_{480nm} (Mean±SD) of *E.coli* ATCC 25922						
	LL-37	LL-37+NaNO2	*P* value*	LL-37+AA	*P* value•	LL-37+AN	*P* value†
2	0.252±0.010	0.436±0.011	<0.001	0.323±0.005	0.001	0.200±0.020	0.02
4	0.233±0.011	0.396±0.015	<0.001	0.293±0.011	0.003	0.156±0.011	0.001
8	0.236±0.020	0.373±0.015	0.001	0.273±0.011	0.05	0.140±0.036	0.02
16	0.213±0.152	0.293±0.011	0.002	0.236±0.005	0.06	0.062±0.020	0.001
32	0.173±0.005	0.306±0.011	<0.001	0.223±0.015	0.06	0.060±0.010	<0.001
64	0.126±0.011	0.300±0.017	<0.001	0.203±0.015	0.002	0.053±0.005	0.001

*** Comparing (LL-37+$NaNO_2$) with LL-37**
• Comparing (LL-37+AA) with LL-37
† Comparing (LL-37+AN) with LL-37

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Discussion

Reactive nitrogen ($NaNO_2$) is an oxidizing compound that causes bacterial death through protein denaturation.[12] The present study reveals that the antibacterial effect of $NaNO_2$ is enhanced by the addition of acidified agents. Indeed, this defence process is known to operate in the human body as part of the overall immune mechanisms. The antibacterial effect of $NaNO_2$ is dependent on physiological variables, such as diet and the level and type of endogenous nitrates. Nitrate in human saliva is converted to nitrite, and this conversion is enhanced by nitrate-reducing bacteria in the mouth.[13] Nitrite that passes from the mouth into the stomach is also reduced by the acidic pH of the stomach to become reactive nitrogen species that have antimicrobial activity against a wide range of gastrointestinal pathogens.[14] Similarity, nitrate excreted through urine and also during infections with nitrate-reducing bacteria, is also reduced to nitrite. At a low pH, nitrite is converted to a variety of nitrogen oxides that are toxic to bacteria.[10] The present study shows that LL-37 has antibacterial activity when used alone or in combination with the other test compound. This antibacterial activity was dose-dependent between the concentrations of 2 to 64µg/ml. The antibacterial activity of LL-37 is affected through electrostatic interactions between cationic domains of LL-37and negatively charged structure in the plasma membrane of bacteria. The cationic domains of LL-37 disrupts the normally protective bacterial plasma membrane.[15,16] The study also examined the effect of acidity on the activity of human cathelicidin LL-37. The results revealed that reducing pH by ascorbic acid diminished the ability of LL-37 to inhibit the growth of both S. aureus ATCC 25923 and E.coli ATCC 25922. This data indicates that a reduced pH inhibits the antibacterial effects of LL-37 and may, therefore, impair host defense mechanisms. These results are consistent with findings by Bechinger (1996) and Aisenbrey et al. (2008)[17,18] who revealed that at alkaline pH, LL-37 has a lower positive charge and thus is inserted more quickly into the bacterial membrane. More recent studies indicate that LL-37 has a broad antimicrobial spectrum against S. aureus and P. aeruginosa at neutral pH, while its antibacterial activity is inhibited at low pH conditions.[19,20] Data depicted in Figures 1 and 2 show weak antibacterial activity for AN as the means±SD of OD_{480nm} for the bacterial growth is increased in comparison with either $NaNO_2$ or AA when used alone. Indeed, the formation of small amounts of reactive nitrogen species could be generated by small amounts of AN and this encourages bacteria to induce cadaverine, which increases bacterial survival through stimulating polyamine production and this, in turn, promoted better bacteria growth.[21] Combining the sub-MIC of AN with a range of concentrations of LL-37 was also assessed in the study. Thus, the study was designed so as to test sub-MIC of AN (10µM of $NaNO_{2+}$ 5mM of AA) at pH 5. Selecting these concentrations was based on the physiological concentrations of the compounds in the human body.[10] The results showed that the combination of LL-37 and AN had a synergistic effect with significantly higher antibacterial activity than either agent alone. The mechanism of this synergistic effect is unknown but is possibly due to the reducing effect of LL-37. This reducing effect increases the production of nitric oxide from $NaNO_2$.[22]

Conclusion

This study demonstrates that the use of LL-37 with AN together has a synergistic effect in comparison with the bacterial effect of either compound when used alone. Therefore, agents that show little antibacterial activity can contribute to host defences when combined with others during episodes of infection.

Conflicts of interest

The author reports no conflicts of interest.

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