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Modulation of exogenous antibiotic activity by host cathelicidin LL-37

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Abstract

The increasing number of infections caused by drug-resistant bacteria has spurred efforts to develop new therapeutic strategies. When applied locally, exogenous antibiotics work in an environment rich in endogenous antibacterial molecules such as the cathelicidin peptide LL-37, which has increased expression at infection sites because of the stimulatory effects of bacterial wall products on neutrophils and other cell types. To test for possible additive effects of exogenous and endogenous antibacterial agents, we evaluated the minimal inhibitory concentration (MIC) to assess the antibacterial activity of amoxicillin with clavulanic acid (AMC), tetracycline (T), erythromycin (E) and amikacin (AN) against different clinical isolates of *Staphylococcus aureus* in combination with synthetic LL-37. These studies revealed that the antibacterial activity of AMC was strongly potentiated when added in combination with LL-37. However, in the presence of LL-37, we did not observe any decrease in the MIC values of T and E, particularly against methicillin-resistant *S. aureus* and macrolide-lincosamide-streptogramin B (MLS_B) (+)/β-lactamase (+) strains, indicating a lack of synergistic action between these molecules. Interaction between exogenous antibiotics and host antibacterial molecules should be considered to provide optimal treatment, especially in cases of topical infections accompanied by increasing expression of host antibacterial molecules.

Keywords

Antibiotics; bacteria; human; cathelicidin LL-37

Natural cationic antimicrobial peptides (CAMPs) form a first line of defense against different pathogens at the epidermal and mucosal epithelial surfaces. As bacteria, viruses and fungi first penetrate the host immune system via epidermal and mucosal epithelia, and because most topical infections occur in the area of pathogen entrance, CAMPs play a significant role in host innate immune defense to prevent and fight infections (1–4). LL-37, a major human cationic antimicrobial peptide, is produced by protease-3-mediated cleavage

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TRANSPARENCY DECLARATION

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(5) from the C-terminal of human cathelicidin (hCAP-18), a protein expressed mostly by neutrophils and epithelial cells. LL-37 is produced constitutively and is found at mucosal surfaces and most body fluids at a concentration of 0.2–1.5 μM , but at infection sites the concentration can increase more than 10 times (6). An experimentally induced increase in LL-37 concentration in airway surface fluid of cystic fibrosis xenografts exposed to adenovirus expressing hCAP-18/LL-37 was sufficient to restore bacterial killing to normal levels, providing strong evidence that the production of antimicrobial peptides is sufficient to protect against bacterial infection (7). In addition to its direct bacterial killing activity, LL-37 prevents bacterial biofilm formation (5), inactivates bacterial lipopolysaccharide (LPS) (8), modulates various immune responses (9) and promotes wound healing (10). Many factors can interfere with LL-37 antibacterial activity, with apolipoprotein A-I (11, 12) as well as negatively charged biopolymers (13) and glycosaminoglycans (14) being the dominant host inhibitors of LL-37 activity in the blood and other biologic fluids, respectively. Some bacterial strains express high levels of aureolysin that can cause cleavage and inactivation of LL-37 (15).

Although LL-37's effects are additive, but not synergistic with those of other endogenous antimicrobial factors in airway fluid (16), and show a synergistic effect with β -defensin (17) and lysozyme against *Staphylococcus aureus* and *Escherichia coli in vitro* (18), less is known about how LL-37 works in combination with exogenous antibiotics. In this study, we evaluate the combined action of four different antibiotics together with LL-37 against different clinical strains of *S. aureus*. The decreased minimal inhibitory concentration (MIC) of some exogenous antibiotics when combined with LL-37 could be beneficial in the treatment of bacterial infection and should be considered in optimizing antibiotic therapy.

MATERIALS AND METHODS

Materials

Mueller–Hinton agar (MHA) was purchased from Difco (Sparks, MD, USA). An ID 32 STAPH Kit to identify staphylococcal isolates and mannitol salt agar (MSA) were from bioMérieux (La Balme Les Grottes, France). E-test kits to determine susceptibility to methicillin were obtained from AB Biodisk (Solna, Sweden). β -lactamase (cefinaise) test kit was from Becton Dickinson (San Jose, CA, USA). LL-37 was purchased from Peptide 2.0 Inc. (Chantilly, VA, USA).

Antimicrobial testing

Bacteria from clinical specimens were grown on MHA. *Staphylococcus aureus* identification was carried out using an ID 32 STAPH Kit followed by reading of results using the ATB system from bioMérieux according to the manufacturer's instructions. Susceptibility to methicillin and vancomycin, and the presence of β -lactamase were determined using an E-test and a cefinaise test, respectively. Susceptibility of *S. aureus* to macrolides, lincosamides and streptogramin B was evaluated using diffusion methods on MHA with bacterial inoculum density corresponding to 0.5 on the McFarland scale (19, 20). MIC and minimal bactericidal concentration (MBC) of LL-37 against different strains of *S. aureus* (8×10^5 cfu/mL) were determined using Müller–Hinton broth and MHA, respectively. Combined antibacterial activity was determined with the use of serial dilutions of two antibacterial agents mixed together in microtitre plates so that each row and column of the plate contained a fixed amount of one agent and an increasing concentration of the other (a standard checkerboard assay). The concentration ranged from the value approximately equivalent to the MIC to that of an eightfold dilution. Each plate also contained a row and column in which a serial dilution of each agent was presented alone. Antibiotic synergism was evaluated using criteria A (two agents were considered synergistic when the growth was

inhibited by a combination of each agent at <25% of the amount required for each agent to inhibit growth alone) and B (if $S < 1$ in the equation $S = (A_c/A_e) + (B_c/B_e)$, with A_e and B_e being the concentrations of LL-37 and an antibiotic when used alone, and A_c and B_c being the concentrations of the two agents in combination). If the sum (S) was <1, the combination was synergistic; if $S > 1$ the combination was antagonistic; and if equal, then the combination was indifferent. If the sum was >1 for some combinations of concentrations and <1 for others, the results were considered equivocal (21).

RESULTS

Clinical isolates of *Staphylococcus aureus*

From the group of 20 clinical isolates of *S. aureus* isolated from pus (diabetic foot or infected surgical wounds), based on their susceptibility to methicillin, macrolides, lincosamides and streptogramin B, and the presence of β -lactamase (data not shown), six different clinical strains were chosen and subjected to evaluation of susceptibility to amoxicillin with clavulanic acid, tetracycline, erythromycin and amikacin (Table 1). One laboratory strain of *S. aureus* ATCC 29213 was also evaluated. In the group of clinical isolates, strains 2, 6 and 7 show intermediate or high resistance to methicillin (MIC of 32–512 or 1024 $\mu\text{g/mL}$, respectively) and streptogramin B. All *S. aureus* strains expressed β -lactamase, but were susceptible to vancomycin. All selected strains had similar LL-37 MIC values of 3.1 μM , and their LL-37 MBC values ranged between 6.25 and 12.5 μM .

Amoxicillin, tetracycline, erythromycin and amikacin activity in the presence of LL-37

Traditional treatment for *Staphylococcus*, including methicillin-resistant *S. aureus* (MRSA), infection involves the use of locally applied antibiotic ointment and/or oral antibiotics. For the best treatment outcome, antibiotic choice should be based on an antibiotic sensitivity test. However, the susceptibility test provides only information about the activity of individual antibiotics and does not take into account the possible interaction between the applied antibiotics and host defense molecules during treatment. Data shown in Table 2 indicate that the local action of exogenous antibiotics can be significantly augmented by host antibacterial molecules such as LL-37. All MIC values determined for amoxicillin with clavulanic acid (a bactericidal member of the β -lactam antibiotic family that, as an alternative substrate for transpeptidases, prevents cross-linking of peptidoglycan, which inhibits bacterial cell wall biosynthesis) were lower in the presence of LL-37 at a concentration that is eight times below the LL-37 MBC value. Similarly, MIC values of amikacin (an aminoglycoside that inhibits bacterial protein synthesis when bound to the 30S ribosomal subunit) against strains 1, 3, 4 and 5 were lower when amikacin was added to the bacteria in combination with LL-37. However, a combination of LL-37 with tetracycline (a bacteriostatic molecule that inhibits binding of aminoacyl tRNA to the 30S ribosomal subunit) and erythromycin (a macrolide that binds to the ribosomal subunit 50S and inhibits peptidyl transferase activity) showed lower effects against strains 2, 5, 6 and 7, compared with individual antibiotic activity. Evaluation of these data using criteria A and B confirms a synergy between the combination of amoxicillin with clavulanic acid and LL-37 (Table 3). These results suggest that exogenous bactericidal antibiotics, especially those that alter bacterial wall structure, can provide a therapeutic advantage when applied in the presence of natural host CAMPs.

DISCUSSION

Data analysis collected by the Surveillance Network, USA (TSN), reveals that *S. aureus* was the most prevalent species isolated from inpatient specimens (18.7% of all bacterial isolates) and the second most prevalent (14.7%) from outpatient specimens (22). The focus on

infections has increased as more and more patients currently admitted to hospitals present community-acquired antibiotic-resistant bacteria, such as certain strains of MRSA, which previously were found predominantly in hospitals and other health care settings. There is a substantial increase in mortality, morbidity and health care cost concerning patients with infections caused by antibiotic-resistant compared with antibiotic-susceptible strains (23). Even though exogenous antibiotics have been used for many decades, better understanding of factors that interfere with their activity is still needed to provide optimal treatment and to develop new strategies to treat bacterial infection, especially those caused by drug-resistant strains. Synergy, antagonism or additive effects on bacterial growth and survival represent the possible outcomes of combined antibiotic therapy that is mostly determined by the mechanism of action of individual agents in the therapeutic regimen. In general, synergy and additive effects are observed when bactericidal or bacteriostatic antibiotics are applied together, whereas a combination of bactericidal with bacteriostatic antibiotics can result in antagonism. Since most host defense antibacterial peptides, including LL-37, kill bacteria as a result of changes in the structure of the bacterial membrane, thus causing dysfunction of its permeability barrier, it is plausible that exogenous antibiotics applied together with LL-37 can result in synergy when the exogenous antibiotic is chosen from the bactericidal family. Our data support this hypothesis; antibacterial activity of amoxicillin with clavulanic acid, and amikacin (both bactericidal), but not that of tetracycline and erythromycin (both bacteriostatic), was observed to increase in combination with LL-37. LL-37-mediated sensitization to exogenous antibiotics can be potentially explained by membrane permeabilization, which facilitates antibiotic entry into bacterial cells to reach their targets (24). However, such a mechanism cannot explain preferential cooperation with bactericidal antibiotics, and it is possible that bacteria exposed to antibiotics that compromise bacterial wall structure become more susceptible to endogenous CAMPs including LL-37. Combinations of bacteriostatic with bactericidal antibiotics against bacterial strains characterized by low susceptibility usually result in an antagonistic effect. In our study, the MIC of tetracycline and erythromycin against three clinical isolates of multidrug-resistant *S. aureus* increased in the presence of LL-37, suggesting antagonism of their combined action. The bacterial stress response that occurs when bacteria are exposed to bacteriostatic antibiotics might provide a mechanism to explain the decrease in their susceptibility to LL-37, which in our results is manifested as an increase in exogenous antibiotic MIC. It can be speculated that bacteria treated with bacteriostatic antibiotics might increase the expression of multidrug resistance efflux pumps. These energy-dependent pumps will act to expel LL-37 to decrease its activity (25). In general, as a result of combined antibacterial therapy of LL-37 with bacteriostatic or bactericidal antibiotics, antagonism and synergy may be expected, respectively.

In summary, our data provide experimental evidence that in the course of antibiotic therapy, antibacterial drugs can interact with host CAPs, and these interactions should be taken into consideration. As bactericidal antibiotics are likely to result in an additive effect with LL-37, this combination might provide a therapeutic advantage to fight infection.

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ABBREVIATIONS

AMC	amoxicillin with clavulanic acid
AN	amikacin

CAMPs	cationic antimicrobial peptides
E	erythromycin
hCAP-18	human cathelicidin
MBC	minimal bactericidal concentration
MHA	Mueller–Hinton agar
MIC	minimal inhibitory concentration
MSA	mannitol salt agar
T	tetracycline

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Antibacterial activity of amoxicillin with clavulanic acid, 2/1 (AMC; S 4 µg/mL, R 8 µg/mL), tetracycline (T; S 4 µg/mL, I = 8 µg/mL, R 16 µg/mL), erythromycin (E; S 0.5 µg/mL, I = 1–4 µg/mL, R 8 µg/mL), amikacin (AN; S 16 µg/mL, I = 32 µg/mL, R 64 µg/mL) and LL-37 against seven different strains of *Staphylococcus aureus*

Table 1

Bacterial strain	MIC (µg/mL)		MIC/MBC of LL-37 (µM)	
	AMC	T	E	AN
<i>S. aureus</i> ATCC 29213 MSSA, MLS _B (-), β-lactamase (+) (Strain 1)	0.4 (S)	1.25 (S)	0.4 (S)	1.6 (S)
				3.125/6.25
<i>S. aureus</i> (clinical strain) MRSA, MLS _B (+), β-lactamase (+) (Strain 2)	12.5 (R)	0.625 (S)	400 (R)	25 (I)
				3.125/12.5
<i>S. aureus</i> (clinical strain) MSSA, MLS _B (-), β-lactamase (+) (Strain 3)	0.2 (S)	1.25 (S)	0.2 (S)	1.6 (S)
				3.125/6.25
<i>S. aureus</i> (clinical strain) MSSA, MLS _B (-), β-lactamase(+) (Strain 4)	1.6 (S)	1.25 (S)	0.4 (S)	3.2 (S)
				3.125/6.25
<i>S. aureus</i> (clinical strain) MSSA, MLS _B (-), β-lactamase (+) (Strain 5)	3.2 (S)	40 (R)	0.2 (S)	1.6 (S)
				3.125/6.25
<i>S. aureus</i> (clinical strain) MRSA, MLS _B (+), β-lactamase (+) (Strain 6)	12.5 (R)	20 (R)	400 (R)	25 (I)
				3.125/6.25
<i>S. aureus</i> (clinical strain) MRSA, MLS _B (+), β-lactamase (+) (Strain 7)	25 (R)	2.5 (S)	200 (R)	1.6 (S)
				3.125/6.25

MRSA, methicillin-resistant *S. aureus*; MSSA, methicillin-sensitive *S. aureus*; S, sensitive; R, resistant; I, intermediate; MIC, minimal inhibitory concentration; MBC, minimal bactericidal concentration.

Antibacterial activity (minimal inhibitory concentration or MIC) of amoxicillin with clavulanic acid (2/1; AMC), tetracycline (T), erythromycin (E) and amikacin (AN) against seven selected strains of *Staphylococcus aureus* (please see Table 1 for strain characteristics) in combination with LL-37. Numerical values represent antibiotic concentrations (µg/mL). Minimal bactericidal concentration values of LL-37 are indicated by the bold numbers in the first left column. For each bacterial strain, a positive control of bacterial growth without an antibacterial agent was performed

Table 2

Bacterial strain	Antibiotic	MIC of antibiotic (µg/mL)	MIC of antibiotic with LL-37 (concentrations in µM)					
			0.8	1.6	3.125	6.25	12.5	
Strain 1 (6.25 µM)	AMC	0.4	0.2	0.1	0.05			
	T	1.25	1.25	1.25	0.312			
	E	0.4	0.4	0.4	0.05			
	AN	1.6	1.6	1.6	0.2			
Strain 2 (12.5 µM)	AMC	12.5	12.5	6.25	3.125	1.6		
	T	0.625	> 0.625	> 400	0.625	0.08		
	E	400	> 400	> 400	> 400	50		
	AN	50	> 50	50	25	12.5	6.25	
Strain 3 (6.25 µM)	AMC	0.4	0.2	0.2	0.1	0.05		
	T	1.25	1.25	1.25	1.25	0.16		
	E	0.2	0.2	0.2	0.2	0.025		
	AN	1.6	1.6	1.6	1.6	0.2		
Strain 4 (6.25 µM)	AMC	1.6	0.4	0.2	0.2			
	T	1.25	1.25	1.25	0.16			
	E	0.4	0.4	0.05				
	AN	3.2	3.2	1.6	0.4			
Strain 5 (6.25 µM)	AMC	3.125	1.6	0.8	0.4	0.4		
	T	40	> 40	> 40	> 40	5		
	E	0.2	> 0.2	> 0.2	0.025			
	AN	1.6	1.6	1.6	0.8	0.2		
Strain 6 (6.25 µM)	AMC	12.5	3.125					
	T	20	> 20	> 20	20	2.5		
	E	400	> 400	> 400	> 400	50		
	AN	25	> 25	> 25	3.125			

Bacterial strain	Antibiotic	MIC of antibiotic (µg/mL)	MIC of antibiotic with LL-37 (concentrations in µM)				
			0.8	1.6	3.125	6.25	12.5
Strain 7 (6.25 µM)	AMC	50	6.25				
	T	2.5	> 2.5	> 2.5	0.312		
	E	200	> 200	> 200	25		
	AN	1.6	> 1.6	> 1.6	0.2		

Table 3

Antibiotic synergism for different concentrations of antibiotics and LL-37 was evaluated using Criteria A and B (please see description in 'Methods' section)

Bacterial strain	Antibiotic	Checkerboard assay	
		Criterion A	Criterion B
Strain 1	AMC	S	S
	T	O	E
	E	O	E
	AN	O	E
Strain 2	AMC	O	E
	T	O	E
	E	O	E
	AN	O	E
Strain 3	AMC	S	S
	T	O	E
	E	O	E
	AN	O	E
Strain 4	AMC	S	S
	T	O	E
	E	O	E
	AN	O	S
Strain 5	AMC	S	E
	T	O	A
	E	O	E
	AN	O	E
Strain 6	AMC	S	S
	T	O	A
	E	O	A
	AN	O	E
Strain 7	AMC	S	S
	T	O	E
	E	O	E
	AN	O	E

AMC, amoxicillin with clavulanic acid, 2/1; T, tetracycline; E, erythromycin; AN, amikacin; For checkerboard assay: S, synergy; O, no synergy; A, antagonism; E, equivocal.