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In Vitro Activities of Tritrpticin Alone and in Combination with Other Antimicrobial Agents against *Pseudomonas aeruginosa*▽

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The in vitro activity of the cathelicidin tritrpticin was investigated against multidrug-resistant *Pseudomonas aeruginosa*. The isolates were susceptible to the peptide at concentrations of 0.50 to 8 mg/liter. Tritrpticin completely inhibits lipopolysaccharide procoagulant activity at a 10 μM concentration. Fractionary inhibitory concentration indexes (0.385, 0.312, and 0.458) demonstrated synergy between the peptide and β-lactams.

Morbidity and mortality from *Pseudomonas* spp. infections, remain high despite the availability of antibiotics to which the microorganism is sensitive (2, 3, 15). Moreover, exposure of gram-negative organisms to antibacterial agents can also result in endotoxin release and septic shock (4, 12, 13, 22).

Antimicrobial peptides are recognized as an important component of the nonspecific host defense system against invading pathogens (1, 11, 12). Typically, these peptides are relatively short, positively charged, and amphiphilic and are reported to be active against bacteria, fungi, viruses, and protozoa (10–12, 17). They bind to the negatively charged residues of lipopolysaccharide (LPS) of the outer membrane by electrostatic and hydrophobic interactions and so determine the key mechanistic step in the killing of gram-negative organisms (8, 9, 12). Cathelicidins are characterized by conserved propeptide sequences and comprise a family of antimicrobial peptides that have been identified in epithelial tissues and some myeloid cells of humans and animals (23).

Tritrpticin, a member of the cathelicidin family, is a 13-amino-acid antimicrobial peptide. The primary structure of tritrpticin is remarkable because of its high content of Arg (30%), Trp (23%), and Pro (15%). Trp and Pro residues are known to play important roles in the assembly and structure of membrane proteins (18, 19).

In this study we investigated the in vitro activities of tritrpticin alone and in combination with six clinically used antimicrobial agents against several multidrug-resistant strains of *Pseudomonas aeruginosa* isolated from wound infections, bronchoalveolar lavage, or blood of hospitalized patients.

Organisms. Twenty nosocomial isolates of *P. aeruginosa* cultured from hospitalized patients with infection admitted to the Ospedali Riuniti of Ancona, Italy, from January 2004 to December 2005 were tested. *P. aeruginosa* ATCC 27853 was used as a quality control strain.

Agents. Tritrpticin (VRRFPWWWPFLRR), amikacin, colistin (all three from Sigma-Aldrich, Milan Italy), ciprofloxacin (Bayer, Milan, Italy), ceftazidime (GlaxoSmithKline, Verona, Italy), imipenem (Merck, Sharp, and Dohme, Milan, Italy), and piperacillin-tazobactam (TZP) (Wyeth-Lederle, Aprilia, Italy) were diluted in accordance with the manufacturers' recommendations.

LPS-binding assay. A quantitative chromogenic *Limulus* amebocyte assay was performed using a QCL-1000 kit (BioWhittaker, Walkersville, Md.) as described previously (8). The change in optical density (ΔOD) was calculated for the control sample, which contained the peptide with no LPS, and this value was subtracted from the ΔOD for samples containing both the peptide and LPS. Percent peptide-LPS binding was calculated from the quotient (Q) of the ΔOD with peptide divided by the ΔOD peptide-free controls, using the following formula: $(1 - Q) \times 100$. Standard curves generated with increasing amounts of LPS were linear between 0.1 and 1.0 endotoxin units/assay.

MIC and minimal bacterial concentration (MBC) determinations were performed according to the procedures outlined by the Clinical and Laboratory Standards Institute (formerly NCCLS) (16). Experiments were performed in triplicate.

Bacterial killing assay. *P. aeruginosa* ATCC 27853 was grown at 37°C in Mueller-Hinton (MH) broth. Aliquots of exponentially growing bacteria were resuspended in fresh MH broth at approximately 10^7 cells/ml and separately exposed to each peptide at 2× MIC for 0, 10, 20, 30, 60, 120, 240, 480, and 720 min at 37°C. After these times, samples were serially diluted and plated onto MH agar plates to obtain viable colonies.

Synergy studies. In interaction studies, *P. aeruginosa* ATCC 27853 and the 20 clinical strains were used to test the antibiotic combinations by a checkerboard titration method using 96-well polypropylene microtiter plates. The fractionary inhibitory concentration (FIC) indexes were interpreted as follows: <0.5, synergy; 0.5 to 4.0, indifferent; and >4.0, antagonism. In addition, time-kill synergy studies were performed at recommended subinhibitory concentrations (one-fourth and one-half the MIC). Synergy or antagonism was defined as a100-fold increase or decrease, and indifference was defined as a less than 10-fold increase or decrease in killing after incubation with the combination compared to the killing activity of the most active single agent (7).

To evaluate LPS-binding activity, colistin, a peptide antibiotic known to bind LPS with high affinity, was used as a positive

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TABLE 1. MICs and MBCs of tritrpticin and other clinically used antibiotics for 20 clinical isolates of *P. aeruginosa*

Antibiotic	MIC (μg/ml)			MBC (μg/ml)		
	Range	50%	90%	Range	50%	90%
Tritrpticin	1–8	2	8	0.5–32	4	16
Colistin	0.5–8	4	8	1–32	8	16
TZP	4–>256	16	256	8–256	32	>256
Ceftazidime	1–256	16	128	8–256	32	256
Imipenem	0.50–64	4	32	1–128	16	64
Amikacin	1–64	2	16	1–64	4	32
Ciprofloxacin	0.50–32	4	16	1–64	8	64

TABLE 2. Results of interaction studies between tritrpticin and other drugs[a]

Antibiotic	Tritrpticin FIC index (range)[b]
Colistin	0.927 (0.750–1.250)
TZP	0.312 (0.187–0.500)
Ceftazidime	0.385 (0.312–0.500)
Imipenem	0.458 (0.312–0.500)
Amikacin	1.833 (1.500–2.000)
Ciprofloxacin	1.500 (1.000–2.000)

[a] The ranges of concentrations tested were: 0.125 to 64 mg/liter for tritrpticin and 0.25 to 256 mg/liter for the other antimicrobial agents.
[b] The FIC indexes were interpreted as follows: <0.5, synergy; 0.5 to 4.0, indifferent; and >4.0, antagonism (12).

control (6). Tritrpticin binds LPS in the low-micromolar range of peptide concentrations and completely inhibits LPS procoagulant activity at a 10 μM concentration. Compared to colistin on a molar basis, it showed an approximately fivefold lower inhibition activity (50% effective concentrations of 0.40 μM and 1.8 μM for colistin and tritrpticin, respectively).

All *P. aeruginosa* organisms were inhibited by tritrpticin at concentrations of 0.5 to 8 mg/liter. In contrast, high rates of resistance to the clinically used antibiotics were demonstrated. The results are summarized in Table 1. As shown in the same table, the good activity of tritrpticin was confirmed by the MBCs (range of 0.5 to 32 mg/liter), which are comparable to the MBC of colistin and lower than values for other antibiotics.

Killing by tritrpticin was shown to be very rapid: its activity against the organism was complete after a 30-min exposure period at a concentration of 2× MIC (Fig. 1). Colistin showed killing activity that was slightly slower than tritrpticin (60 min). In contrast, as expected, the clinically used antibiotics demonstrated a killing activity completed only after 240 min for amikacin and 480 min for the other agents.

FIC indexes of 0.385, 0.312, and 0.458 were observed by testing tritrpticin combined with ceftazidime, TZP, and imipenem, respectively, against all organisms tested, while the combination of tritrpticin with colistin gave a value of 0.927 with a range of 0.750 to 1.250. The results are summarized in Table 2. These data were confirmed by the time-kill synergy studies (data not shown).

Our data demonstrate that tritrpticin has a powerful antimicrobial and bactericidal effect on multiresistant clinical isolates of *P. aeruginosa*. Its activity is comparable to that of colistin and stronger than the activity of the other clinically used antibiotics. In addition, it completely inhibits LPS procoagulant activity at a 10 μM concentration although, compared to colistin on a molar basis, it exhibited an approximately fivefold lower inhibition activity.

Interaction studies suggest that tritrpticin could be usefully administered in combinations with β-lactam antibiotics to treat severe gram-negative bacterial infections. The cationic peptides allow maximal entry of several substrates inside the cell: the synergistic interaction with β-lactam antibiotics could be due to their increased passage through the outer bacterial membrane (5, 20). On the other hand, peptides and β-lactams may have a common target: it has been hypothesized that cationic peptides might render bacteria nonviable by activating their autolytic wall enzymes, such as muramidases (14, 21, 22).

The intrinsic antibacterial activity and the synergistic interactions demonstrated with several combinations make tritrpticin potentially valuable as an adjuvant for treatment of *P. aeruginosa* infection. Further research toward this aim based on animal models is needed.

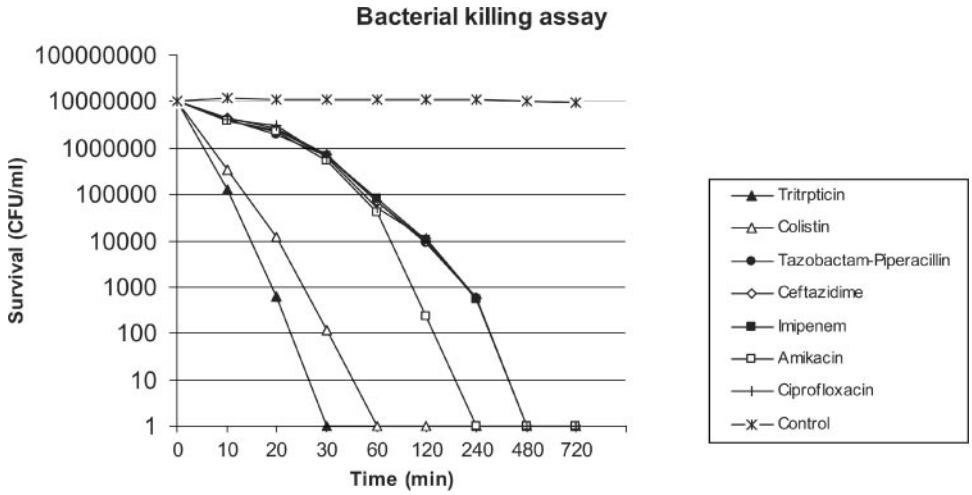

FIG. 1. Time-kill kinetics of tritrpticin and six antibiotics against *P. aeruginosa* ATCC 27853.

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