In Vitro Activities of Tritrpticin Alone and in Combination with Antimicrobial Agents Against *Pseudomonas aeruginosa*

Oscar Cirioni, Andrea Giacometti*, Carmela Silvestri, Agnese Della Vittoria, Alberto Licci, Alessandra Riva, and Giorgio Scalise

*Institute of Infectious Diseases and Public Health, Università Politecnica delle Marche, Ancona, Italy*

Corresponding author:
Andrea Giacometti, M.D., Clinica Malattie Infettive c/o Ospedali Riuniti Ancona via Conca 71 I - 60020 Ancona Italy

Tel: +39-071-5963467
Fax: +39-071-5963468
e.mail: anconacmi@interfree.it or a.giacometti@univpm.it

**Running title:** Tritrpticin and *Pseudomonas aeruginosa*
ABSTRACT

The in vitro activities of the cathelicidin tritpticin was investigated against multidrug-resistant *Pseudomonas aeruginosa*. The isolates were susceptible to the peptide at concentrations of 0.50-8 mg/l. Tritpticin completely inhibits the LPS procoagulant activity at 10 µM concentration. FIC indexes (0.385, 0.312 and 0.458) demonstrated synergy between the peptide and betalactams.

Key Words: Tritpticin, *P. aeruginosa*, antimicrobial activity, antiendotoxin activity
Morbidity and mortality from *Pseudomonas* spp. infections, remains high despite the availability of antibiotics to which the microorganism is sensitive (2,3,16). Moreover, exposure of Gram-negative organisms to antibacterial agents can also result in endotoxin release and determine septic shock (13,14,22).

Antimicrobial peptides are recognized as an important component of the non-specific host defence system against invading pathogens (1,12,13). Typically, these peptides are relatively short, positively charged, amphiphilic and are reported to be active against bacteria, fungi, viruses and protozoa (11-13,17). They bind to the negatively charged residues of LPS of the outer membrane by electrostatic and hydrophobic interactions and so determining the key mechanistic step in the killing of gram-negative organisms (9,10,13). 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 *P. 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 were used as quality control strain.
Agents. Tritrpticin (VRRFPWWPFLRR), amikacin and colistin (all from Sigma-Aldrich, Milan Italy), ciprofloxacin (Bayer, Milan, Italy), ceftazidime (GlaxoSmithKline, Verona, Italy), imipenem (Merck, Sharp & Dohme, Milan, Italy), and piperacillin/tazobactam (TZP) (Wyeth-Lederle, Aprilia, Italy) were diluted in accordance with manufacturers’ recommendations.

LPS-binding assay. A quantitative chromogenic Limulus amebocyte assay was performed using the QCL-1000 kit (BioWhittaker, Walkersville, Md., USA) as described previously (9). 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 formula (1-Q) × 100. Standard curves generated with increasing amounts of LPS were linear between 0.1 and 1.0 endotoxin units (EU)/assay.

MIC and MBC determinations were performed according to the procedures outlined by the Clinical and Laboratory Standards Institute (CLSI) (4). Experiments were performed in triplicate.

Bacterial killing assay. P. aeruginosa ATCC 27853 was grown at 37°C in 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 minutes at 37°C. After these times samples were serially diluted and plated onto MH agar plates to obtain viable colonies.

Sinergy studies. In interaction studies, P. aeruginosa ATCC 27853 and the twenty clinical strains were used to test the antibiotic combinations by a checkerboard titration method using 96-well polypropylene microtitre plates. The fractionary inhibitory concentration (FIC) indexes were interpreted as follows: <0.5, synergy; 0.5-4.0,
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 an -100-fold increase or decrease and indifference was defined as a <10-fold increase or decrease in killing after incubation with the combination compared with that of the most active single agent (8).

To evaluate LPS-binding activity, colistin, a peptide antibiotic known to bind LPS with high affinity, was used as a positive control (7). Tritrpticin binds LPS in the low µM range of peptide concentrations and completely inhibits the LPS procoagulant activity at 10 µM concentration. When compared to colistin on a molar basis, it showed an approximately fivefold lower inhibition activity (EC$_{50}$ values of 0.40 µM and 1.8 µM for colistin and tritrpticin respectively).

All _P. aeruginosa_ strains organisms were inhibited by tritrpticin at concentrations of 0.5 to 8 mg/l. Differently, 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 MBC values (range of 0.5 - 32 mg/l) comparable to those of colistin and lower than 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 a killing activity a slightly slower than tritrpticin (60 min). On the contrary, as expected, the clinically used antibiotics demonstrated a killing activity completed only after 240 min (amikacin) and 480 min the other agents.

FIC indexes of 0.385, 0.312 and 0.458 were observed by testing tritrpticin combined with ceftazidime, tazobactam/piperacillin and imipenem, respectively, against all organisms tested, while its combination with colistin showed a value of 0.927 with
range of 0.750 - 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 other clinically used antibiotics. In addition, it completely inhibits the LPS procoagulant activity at 10 μM concentration although, when compared to colistin on a molar basis, it exhibited an approximately fivefold lower inhibition activity.

Interaction studies suggest that it could be usefully administered in combinations with betalactams antibiotics to treat severe gram-negative infections. The cationic peptides allow maximal entry of several substrates inside the cell: the synergistic interaction with betalactam antibiotics could be due to their increased passage through the outer bacterial membrane (6,20). On the other hand, peptides and betalactams may have a common target: it had been hypothesized that cationic peptides might render bacteria non viable by activating their autolytic wall enzymes, such as muramidases (15,22).

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

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References


Figure legend:

Fig. 1. Time-kill kinetics of tritrpticin and six antibiotics against *P. aeruginosa* ATCC 27853
Table 1. MICs and MBCs of tritrpticin and other clinically used antibiotics for 20 clinical isolates

<table>
<thead>
<tr>
<th>Strains (N°)/Agent</th>
<th>MIC (µg/ml)</th>
<th>MBC (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Range</td>
<td>50%</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tritrpticin</td>
<td>1 – 8</td>
<td>2</td>
</tr>
<tr>
<td>Colistin</td>
<td>0.5 – 8</td>
<td>4</td>
</tr>
<tr>
<td>TZP&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4 - &gt;256</td>
<td>16</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>1 – 256</td>
<td>16</td>
</tr>
<tr>
<td>Imipenem</td>
<td>0.50 – 64</td>
<td>4</td>
</tr>
<tr>
<td>Amikacin</td>
<td>1 – 64</td>
<td>2</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>0.50 – 32</td>
<td>4</td>
</tr>
</tbody>
</table>

<sup>a</sup>TZP, tazobactam/piperacillin
Table 2. Results of interaction studies between Tritrpticin and other drugs

<table>
<thead>
<tr>
<th>Drug</th>
<th>FIC Index (Concentration Range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colistin</td>
<td>0.927 (0.750 – 1.250)</td>
</tr>
<tr>
<td>TZP&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.312 (0.187 – 0.500)</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>0.385 (0.312 – 0.500)</td>
</tr>
<tr>
<td>Imipenem</td>
<td>0.458 (0.312 – 0.500)</td>
</tr>
<tr>
<td>Amikacin</td>
<td>1.833 (1.500 – 2.000)</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>1.500 (1.000 – 2.000)</td>
</tr>
</tbody>
</table>

The ranges of concentrations tested were: 0.125-64 mg/l for tritrpticin and 0.25-256 mg/l for the other antimicrobial agents.

The FIC indexes were interpreted as follows: <0.5, synergy; 0.5-4.0, indifferent; and >4.0, antagonism (13).

<sup>a</sup>TZP, tazobactam/piperacillin