Efficacy of Tachyplesin III, Colistin and Imipenem against Multiresistant Pseudomonas aeruginosa Strain

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Running Head: Antibiotic combination and multiresistant P. aeruginosa

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Abstract

An experimental study has been performed to compare the in vitro activity and the in vivo efficacy of tachyplesin III, colistin and imipenem against multiresistant *Pseudomonas aeruginosa* strain. In vitro experiments included MIC determination, time-kill and synergy studies. For in vivo studies, a mouse model of sepsis has been used. Main outcome measures were: lethality, quantitative blood cultures and detection of LPS, TNF-alpha and interleukin-6 (IL-6) plasma levels.

Combinations of tachyplesin III or colistin with imipenem showed in vitro synergistic interaction. Significant increase in efficacy was also observed in vivo: combined-treated groups had significant lower bacteremia when compared to single-treated groups. Tachyplesin III combined with imipenem exhibited the highest efficacy on all main outcome measurements. These results highlight the potential usefulness of these combinations and provide future therapeutic alternative in serious gram-negative infections in the coming years.

Key Words: *P. aeruginosa*, antibiotics, antimicrobial peptides, resistance, sepsis
Introduction

Resistance among bacteria is on the rise, both in the hospital and in the community (3,22). In comparison with gram-positive cocci, for which resistance to a single antibiotic indicates the antibiotic resistance phenotype of interest, multidrug resistance in gram-negative bacilli is difficult to define. Among these resistant bacteria, *Pseudomonas aeruginosa* represent a pathogen with notable virulence characteristics and the ability to exhibit antibiotic resistance. Multidrug resistance in *P. aeruginosa* has been variously defined as resistance to at least 2 or more antibiotics typically used to treat infections with these organisms. These definitions are clearly arbitrary and may be of questionable practical value to a clinician (21,23). Of greater relevance may be a definition of "panresistance," because the complete or almost complete lack of treatment options beta-lactams, carbapenem and fluoroquinolones is an increasingly common and desperate occurrence in hospital setting (23).

Antimicrobial therapy for infections due to these multi-resistant organisms remains a clinical dilemma in hospitalized patients. Strategies for prevention and therapy, include both infection control and modifications in antibiotic use such as antibiotic cycling or rotation, combination therapies and the use of “old” and new drugs for the management of infections with gram-negative bacilli resistant to all other alternatives (4,17).

After the discovery of polymyxins, a number of cationic peptides have been isolated from a wide range of bacterial species, plant and animals (2,13,15). They are a recently emerged class of antibiotics with therapeutic potential. These molecules are important components of the innate immune response in most multi-cellular organisms used by animals to effectively deal with pathogenic microorganisms in their environments (13,15,19). In mammals, peptides are found in circulating phagocytes, where they contribute to the killing of engulfed micro organisms, and in
epithelial surfaces, where they act as a local defense mechanism that protects anatomical compartments from microbial invasion.

Tachyplesins are a group of antimicrobial peptides isolated from horseshoe crabs. Tachyplesin III (KWCFRVCYRGICYRKCR-NH₂) isolated from Southeast Asian horseshoe crabs Tachyleus gigas and Carcinoscorpius rotundicauda, hem lymph consisting of 17 amino acids with two disulfide bridges is a representative antimicrobial peptide with cyclic β-sheet. It is similar in structure and activity profile to protegrins, a family of peptides found in pig intestines. Because of its potency and relatively small sizes, this peptide is an attractive target for structure-activity studies that may lead to therapeutics to treat infections (14,20). Tachyplesin III exhibit a broad-spectrum activity against gram-negative and -positive bacteria, fungi and even enveloped viruses at low concentrations. Furthermore, it has also been shown to play a role in the proinflammatory response because it forms complexes with bacterial lipopolysaccharides that neutralize the factor C-activating activity of LPS in a manner similar to that of anti-LPS factor (20). It was reported, even though the precise action mechanism of the cationic peptides remains to be determined, that they rapidly perturbed the membrane function of pathogenic microorganisms.

Recent reports have shown that synergistic effect was observed in several clinically isolated bacterial strains when some antimicrobial peptides were combined with several clinically used antibiotics. Therefore, the presence of this synergistic effect makes the cationic peptides potentially valuable as an adjuvant for antimicrobial chemotherapy against antibiotic-resistant bacterial strains (10,25).

In order to broaden our knowledge of this role, we evaluated the activity of the combination of tachyplesin III, colistin and imipenem in vitro and in vivo using two P. aeruginosa strains.

Materials and Methods
Organisms

The commercially available quality control strain of *P. aeruginosa* ATCC 27853 and one clinical isolate multiresistant *P. aeruginosa* strain were used in this study. The clinical strain was isolated from a specimen submitted for routine bacteriological investigation to the Institute of Infectious Diseases and Public Health, Polytechnic University of Marche, Ancona, Italy. Its susceptibility pattern indicate resistance to aminopenicillins, aztreonam, cephalosporins, carbapenems, quinolones, and aminoglycosides.

Synthetic peptide

Tachyplesin III (molecular weight 2495.5 Da) was synthesized by 9-fluorenlymethoxycarbonyl (Fmoc) solid-phase chemistry (5,9). The protected peptidyl resin was treated with the mixture: 92% trifluoroacetic acid (TFA), 2% ethanedithiol (EDT), 2% water and 2% triisopropylsilane (TIS) for 2 h. After cleavage the solid support was removed by filtration, and the filtrate was concentrated under reduced pressure. The cleaved peptide was precipitated with diethyl ether, dissolved in 20% acetic acid and oxidized by 0.1 M iodine in methanol. Tachyplesin III was purified and analyzed by high-performance liquid chromatography (HPLC). The resulting fractions with purity greater than 94-95% were tested by HPLC. The peptide was analyzed by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF). The peptide was solubilized in phosphate buffered saline (pH 7.2) yielding 1 mg/ml stock solution. Solutions of drugs were made fresh on the day of assay or stored at -80°C in the dark for short periods.

MIC determination and bacterial killing assay

Tachyplesin III, colistin (Sigma-Aldrich, Milan, Italy) and imipenem (Merck, Sharp & Dohme, Milan, Italy), powders were diluted in accordance with manufacturers’ recommendations. Solutions of drugs were made fresh on the day of assay or stored at -80°C in the dark until twenty days. The
MIC was determined using a microbroth dilution method with Mueller-Hinton (MH) broth (Becton Dickinson Italia, Milan, Italy) and an initial inoculum of $5 \times 10^5$ cfu/mL, according to the procedures outlined by the Clinical and Laboratory Standards Institute (CLSI) (6). Polypropylene 96-well plates (Becton Dickinson and Co., Franklin Lakes, N.J.) were incubated for 18 h at 37°C in air. When peptides were tested, since some of these compounds has a tendency to precipitate, plates were shaken throughout the study. The MIC was taken as the lowest drug concentration at which observable growth was inhibited. Experiments were performed in triplicate. The MBC was taken as the lowest concentration of each drug that resulted in more than 99.9% reduction of the initial inoculum. Experiments were performed in triplicate.

Both strains were 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, 2, 5, 10, 20, 30, 40, 50 and 60 minutes at 37°C. After these times 0.1 mL samples were serially diluted and plated onto MH agar plates to obtain viable colonies. The limit of detection for this method was approximately 10 cfu/mL. In preliminary experiments, antibiotic carryover was ruled out by plating samples of bacterial suspensions. Killing was also investigated in combination studies, to evaluate the activity of imipenem at concentration of 4 mg/L when combined with each peptide at 0.5 × MIC and 1 × MIC. In all tubes a $5 \times 10^5$ cfu/mL log-phase inoculum was added along with MH broth to give a final volume of 10 ml. All tubes were incubated overnight at 35°C and the bacterial growth in each tube was determined by performing consecutive 1:10 (v/v) dilutions of a 0.1 mL aliquot of each tube in MH broth and by plating a 0.1 mL volume of each dilution onto MH agar. Experiments were performed in triplicate. If a combination caused a decrease in viable cell count of ≥ 2 log$_{10}$ compared with the most active single agents, the effects of the combination were considered to be synergic. If the decrease in viable cell count was 1-2 log$_{10}$ the effects of the combination were considered to be additive.

**Synergy studies**
In interaction studies, the strains were used to test the antibiotic combinations by a checkerboard titration method using 96-well polypropylene microtitre plates. The ranges of drug dilutions used were: 0.125-64 mg/L for tachyplesin III and 0.25-256 mg/L for colistin and imipenem. The fractionary inhibitory concentration (FIC) index for combinations of two antimicrobials was calculated according to the equation: \[ \text{FIC index} = \frac{A}{\text{MIC}_A} + \frac{B}{\text{MIC}_B}, \] where \( A \) and \( B \) are the MICs of drug \( A \) and drug \( B \) in the combination, \( \text{MIC}_A \) and \( \text{MIC}_B \) are the MICs of drug \( A \) and drug \( B \) alone, and \( \text{FIC}_A \) and \( \text{FIC}_B \) are the MICs of drug \( A \) and drug \( B \). The FIC indexes were interpreted as follows: The FIC indexes were interpreted as follows: <0.5, synergy; 0.5-4.0, indifferent; and >4.0, antagonism (8). In addition, time-kill synergy studies were performed at recommended sub inhibitory 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.

**Hemolysis of human red blood cells and cytotoxicity assay**

Fresh hRBC with EDTA were rinsed 3 times with PBS (35 mM phosphate buffer/0.15 M NaCl, pH 7.3) by centrifugation for 10 min at 800 x g at 35° and resuspended in PBS. Tachyplesin dissolved in PBS were then added to 50 µL of a solution of the stock hRBC in PBS to reach a final volume of 100 µL (final erythrocyte concentration, 4% v/v). The resulting suspension was incubated with agitation for 60 min at 37 °C. The samples were then centrifuged at 800 x g for 10 min. The release of hemoglobin was monitored by measuring the absorbance of the supernatant at 540 nm. Controls for 0 hemolysis (blank) and 100% hemolysis consisted of hRBC suspended in PBS and Triton 1%, respectively.

For cytotoxicity assay A-549 cells from human lung carcinoma ((BioWhittaker Inc., Walkersville, MD, USA) were cultured in 25 cm² tissue culture flasks. Medium consisted of DMEM with 10%
foetal calf serum (Bio-Whittaker). The cytotoxicities of tachyplesin III A at 1×MBC were
determined by the CellTiter 96 AQ cell proliferation assay (Promega Corp., Lyon, France).

Animals
BALB/c male mice weighting 25 to 30 g were used for all the experiments. Each mouse was housed
in individual cage under constant temperature (22°C) and humidity with 12-hours light/dark cycle,
and had access to chow and water ad libitum throughout the study. The study was approved by the
animal research ethics committee of the I.N.R.C.A. - I.R.R.C.S., Polytechnic University of Marche,
Ancona, Italy.

Preparation and implantation of the inoculum and antibiotic therapy
P. aeruginosa ATCC 27853 and clinical isolate strain were grown overnight at 37°C in brain-heart
infusion broth. When bacteria were in the log phase of growth the suspension was centrifuged at
1000 x g for 15 min, the supernatant was discarded, and the bacteria were resuspended in sterile
saline to achieve a concentration of approximately 1×10^8 CFU/mL. All animals were anesthetized
by an intramuscular injection of ketamine (30 mg/kg of body weight). Mice were injected
intravenously (i.v.) via the tail vein with 0.2 mL of the above mentioned bacterial suspensions: i)
2.0×10^7 cfu of strains on day 0 and monitored for 72 hours.
Immediately after bacterial challenge, the mice were randomized to receive intravenously isotonic
sodium chloride solution (control group), 20 mg/Kg imipenem, 1 mg/Kg tachyplesin III, 1 mg/Kg
colistin, and finally 1 mg/Kg tachyplesin III or 1 mg/Kg colistin combined with 20 mg/Kg
imipenem. Each group included 20 mice. The animals were returned to individual cages and
monitored for the subsequent 72 hours. The endpoints of the study were lethality rates, quantitative
blood cultures, endotoxin, TNF-alpha and IL-6 plasma levels. Toxicity was evaluated on the basis
of the presence of drug related adverse effects (local signs of inflammation, weight loss, vomiting,
diarrhea and fever) in a supplementary peptide-treated groups without challenge.
Evaluation of treatment

Blood samples for culture were obtained from the tail vein by aseptic percutaneous puncture 24 h after bacterial challenge. The animals that died before this time were not tested. To perform quantitative bacterial cultures, blood samples were serially diluted and a 0.1 ml volume of each dilution was spread on blood agar plates and cultured at 35°C for 48 h and the CFU counted. The limit of detection was 10 CFU/mL.

For determination of endotoxin, TNF-alpha and IL-6 in plasma, blood samples (0.2 mL for each animal) were collected from the tail vein after 0, 6, 12, 24 and 48 h post-injection. Endotoxin concentrations were measured by the commercially available Limulus amebocyte lysate test (E-TOXATE®, Sigma-Aldrich). Endotoxin standards (0, 0.015, 0.03, 0.06, 0.125, 0.25, and 0.5 EU/mL) were tested in each run, and the concentration of endotoxin in the tested samples was calculated by comparison with the standard curve.

IL-6 and TNF-alpha levels were measured by ELISA according to the manufacturer’s specification (Walter Occhiena srl, Turin, Italy). The lower limits of sensitivity for IL-6 and TNF-alpha were 12 pg/ml and 0.05 ng/ml, respectively. The assays were performed in duplicate.

Statistical analysis

Lethality rates between groups were compared by use of Fisher’s exact test. Data from quantitative blood cultures were presented as means ± standard deviations (SDs) of the mean; statistical comparisons between groups were made by analysis of variance. Post hoc comparisons were performed by Bonferroni’s test. Plasma endotoxin, IL-6 and TNF-alpha mean values were compared between groups by analysis of variance. Significance was accepted when the p value was ≤0.05.
Results

In vitro studies

According to the broth micro dilution method recommended by the CLSI, imipenem exhibited MICs of 0.50 and 32 mg/L for *P. aeruginosa* ATCC 27853 and the clinical isolate (data not shown). Colistin and tachyplesin III exhibited MICs of 4 and 4 mg/L for *P. aeruginosa* ATCC 27853 and 8 and 4 mg/L for the multiresistant strain, respectively. For both the strains killing by tachyplesin III was shown to be very rapid: its activity against the organism was complete after a 15 min exposure period at a concentration of 2×MIC. Colistin showed the same activity, while, imipenem exhibited a slower bactericidal effect on *P. aeruginosa*. Interestingly, an increase in killing at 60 min greater than 100-fold was observed when the peptides were combined (data not shown). In the combination studies, synergy was observed between tachyplesin III and imipenem both for *P. aeruginosa* ATCC 27853 (FIC indexes 0.312; MICs 2 and 0.12 mg/L for tachyplesin III and imipenem, respectively) and the clinical isolate (FIC indexes 0.312; MICs 2 and 8 mg/L for tachyplesin III and imipenem, respectively). Similarly, synergy was also observed between colistin and imipenem both for *P. aeruginosa* ATCC 27853 (FIC indexes 0.385; MICs 2 and 0.25 mg/L for tachyplesin III and imipenem, respectively) and the clinical isolate (FIC indexes 0.458; MICs 4 and 8 mg/L for tachyplesin III and imipenem, respectively). These data were confirmed by the time kill synergy studies (data not shown).

The cytotoxic effect was practically absent at the concentrations tested. Finally our data revealed that the peptide showed low haemolytic activity in spite of their high activity against the organism. In fact, its haemolytic activity was observed at concentrations higher than MIC values (4.5%, 12.6%, and 26.7% at concentrations of 8, 16, and 32 mg/L, respectively).

In vivo studies
As shown in Table 1, control group C1, showed a 100% lethality rate within 72 hours. In contrast, for both the strains, immediate treatment with drugs demonstrated efficacy significantly higher than controls ($P < 0.05$). For *P. aeruginosa* ATCC 27853 lethality rates of 30%, 30%, and 40% were observed for groups treated with imipenem, tachyplesin III and colistin A, respectively. For the clinical strain, imipenem showed an higher lethality rate (80%) while the same lethality rates were observed for peptide-treated groups (30%). The combination between tachyplesin III or colistin and imipenem showed significantly lowest lethality rates of 5% and 10% for the control strain and 10, and 15% for the clinical isolate, respectively. Quantitative blood culture showed high bacterial numbers both in the ATCC 27853 group ($5.8 \times 10^7 \pm 0.8 \times 10^7$ CFU/ml) and in the clinical isolate group ($7.9 \times 10^7 \pm 2.2 \times 10^7$ cfu/mL), as shown in Table 1. Tachyplesin III demonstrated a good antibacterial activity against ATCC strain reducing the bacterial growth to $3.6 \times 10^3 \pm 0.6 \times 10^3$ cfu/mL, comparable to those of colistin and imipenem ($6.0 \times 10^4 \pm 1.7 \times 10^4$ cfu/mL and $2.8 \times 10^3 \pm 0.4 \times 10^3$ cfu/mL, respectively). When imipenem was combined with tachyplesin III and colistin, the positive interactions produced the lowest bacterial counts ($1.1 \times 10^1 \pm 0.1 \times 10^1$ cfu/mL for tachyplesin III plus imipenem and $4.6 \times 10^1 \pm 0.5 \times 10^1$ for colistin plus imipenem). Against the panresistant strain tachyplesin III and colistin showed antibacterial activity comparable to that against the ATCC strain, while imipenem showed weak activity. Similarly to the previous experiment, when imipenem was combined with tachyplesin III and colistin the positive interaction produced the lowest bacterial counts. Overall, any combined-treated group had significant lower bacterial counts when compared to singly-treated groups ($P < 0.05$).

For both the strains, plasma peak levels of endotoxin, TNF-alpha and IL-6 were observed 6 and 12 h after bacterial challenge, respectively. Tachyplesin III and colistin treatments (alone or combined) resulted in marked decrease ($P < 0.05$) of endotoxin, TNF-alpha and IL-6 plasma levels compared with those of controls and vancomycin-treated groups. (Figure 1-3). The strongest reduction in
endotoxin and cytokines plasma levels was observed in the groups treated with the combinations between tachyplesin III and imipenem.

None of the animals had clinical evidence of drug-related adverse effects, hypersensitivity reactions, and no changes in physiological parameters were observed in the supplementary peptides-treated groups without previous infection.

Discussion

It is well known that *P. aeruginosa* may become resistant to the antibiotic being used to treat the infection and that prior use of a particular antibiotic predicts that *P. aeruginosa* will develop resistance to that antibiotic. The main reasons for this bacterial resistance is thought to be the organism’s low outer membrane permeability to antimicrobial agents, genetic elements capable to carry and transfer diverse antibiotic resistance determinants and, finally, efflux pumps. Due to its potent inflammatory activity and association with sepsis, LPS can be an important virulence factor in *Pseudomonas* infections (1,12).

In the present study, we evaluated the efficacy of the combination between tachyplesin, colistin and imipenem two strains of *P. aeruginosa* with different pattern of susceptibility, first in vitro and then in vivo. We used one susceptible and one multiresistant strain that can be been associated with clinical failure following antibiotic therapy. In time-killing curves and in checkerboard titration method, a synergistic effect between the peptides and imipenem was observed. This synergistic pattern clearly was also observed in the in vivo setting. Previous studies have reported contradictory results about the interaction among peptides and antibiotics (7,10,25,26). The peptide acts by inserting into the cytoplasmic membrane and triggering the activity of bacterial murein hydrolases, resulting in damage or degradation of bacterial membranes, and lysis of the cell. In addition, antimicrobial peptides can induce, by the loss of effective outer
membrane porin channels, a membrane permeability that facilitate the antibiotic penetration and their consequent enhancement activity (7,18,25,27).

As it has been described previously, a sepsis mouse model was able to compare the efficacy among different antibiotic therapies and also the efficacy of a therapy among strains with different susceptibilities. We selected as main outcome measures lethality, quantitative blood cultures and detection of endotoxin, TNF-alpha and IL-6 plasma levels to have different parameters and better define the efficacies of the treatments. We used a short period of treatment of 24 h in order to increase the relevance of the model, since this is the critical period of such an acute infection (16).

In our model, tachyplesin III and colistin showed superior (clinical isolate) or similar (ATCC control strain) antimicrobial activities and comparable lethality rates to those of imipenem. Differently, they demonstrated an higher ability to inhibit the effects of endotoxin and, consequently, the release of cytokines than imipenem.

Finally, the best results on mortality rates and bacteremia were obtained when tachyplesin III was combined with imipenem. This combination was also most effective in decreasing the levels of cytokines, confirming the capacity of the peptides to neutralize membrane components that are the inducers of cytokine activation. Several studies have shown that exposure of Gram-negative organisms to bactericidal agents such as carbapenems can initially result in endotoxin release, even though successively appear more relevant the decrease in endotoxin release due to the inhibitory effect on the bacterial growth (24). Therefore, new drugs able to neutralize molecules released by Gram-negative organisms and block their interaction with specific receptor on immune cells at the beginning of antimicrobial treatment can be an attractive concept. Today the clinically used antimicrobial peptides are the microbial cyclic molecules such as colistin and polymyxin B. Our results emphasized that tachyplesin III, a linear invertebrate peptides, has an in vitro activity and a systemic in vivo efficacy equivalent to colistin.

Few treatment options remain for serious infections caused by multidrug-resistant bacteria. Carbapenems are active against some isolates of P. aeruginosa species and, although, the
polymyxins remain the most consistently effective agents in vitro against this organism, a growing number of reports have documented resistance to this antibacterial (11). In this situation, novel compounds and antibiotic combinations will be necessary to treat infections caused by these multidrug-resistant organisms and to reduce the increasing selection pressure by antibiotics on gram-negative pathogens. One way to overcome the problems of emergence of resistance is the correct use of new antimicrobial compounds and/or combination therapy. In fact, infections with isolates that are susceptible to only one or a few agents may be treated with a single drug, but the risk of progressive resistance must be considered. Specific combination therapy can be used to increase the in vivo activity, to prevent the emergence of drug resistance and to broaden the antimicrobial spectrum. In particular, sub lethal alterations of the gram-negative bacterial outer membrane in combination with the use of antibiotics that, because of resistance, are now ineffective alone may further extend therapeutic opportunities. The good antimicrobial activities toward gram-negative bacteria with high levels of antibiotic resistance and synergistic interactions with imipenem, illustrates additional important attributes that may support tachyplesin III as promising candidate for adjuvant in antimicrobial therapy against multiresistant organisms. Further studies should be performed to address the developability of tachyplesins.

**Acknowledgements:** We wish to express our thanks to Silvana Esposito for her technical assistance.

**References**


TABLE 1. Effect of tachyplesin III, colistin and imipenem in a mouse model of *P. aeruginosa* induced sepsis. Treatment was carried out immediately post bacterial challenge.

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>Lethality* dead/total (%)</th>
<th>Qualitative blood culture positive/total</th>
<th>Quantitative blood culture* (CFU/ml)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><em>P. aeruginosa</em> ATCC 27853</td>
<td><em>P. aeruginosa</em> clinical isolate</td>
</tr>
<tr>
<td>No treatment (control group C1)</td>
<td>20/20 (100)</td>
<td>20/20 (100)</td>
<td>20/20</td>
</tr>
<tr>
<td>TCP III 1 mg/Kg</td>
<td>6/20d (30)</td>
<td>6/20d (30)</td>
<td>7/20d</td>
</tr>
<tr>
<td>COL 1 mg/Kg</td>
<td>8/20d (40)</td>
<td>6/20d (30)</td>
<td>8/20d</td>
</tr>
<tr>
<td>IMP 20 mg/Kg</td>
<td>6/20d (30)</td>
<td>16/20 (80)</td>
<td>6/20d</td>
</tr>
<tr>
<td>TCP III 1 mg/Kg</td>
<td>1/20de (5)</td>
<td>2/20de (10)</td>
<td>1/20de</td>
</tr>
<tr>
<td>IMP 20 mg/Kg</td>
<td>0/20&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0/20&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2/20&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------------</td>
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</tr>
<tr>
<td>COL 1 mg/Kg</td>
<td>2/20&lt;sup&gt;de&lt;/sup&gt;</td>
<td>3/20&lt;sup&gt;de&lt;/sup&gt;</td>
<td>2/20&lt;sup&gt;de&lt;/sup&gt;</td>
</tr>
<tr>
<td>IMP 20 mg/Kg</td>
<td>0/20&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1/20&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2/20&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>TCP III, tachyplesin III, COL, colistin, IMP, imipenem.

<sup>b</sup>Mortality was monitored for 72 h following the challenge. <sup>b2</sup>Mortality within 24 h.

<sup>c</sup>Mean ± S.D.

<sup>d</sup>P < 0.05 (Fisher’s test) or P < 0.05 (Bonferroni’s test) versus the control group C.

<sup>e</sup>P < 0.05 (Fisher’s test) or P < 0.05 (Bonferroni’s test) versus the singly-treated group.
FIGURES 1-3 LEGEND PAGE

Effects on endotoxin, TNF-alpha and interleukin-6 plasma levels of drugs alone or combined after bacterial challenge. (a) ATCC 27853, (b) multiresistant strain.

Figure 1a
Effect of drug administration after ATCC strain challenge

![Graph showing TNF plasma levels over time for different treatments.]

Figure 1b

Effect of drug administration after multiresistant strain challenge

![Graph showing TNF plasma levels over time for different treatments.]

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Figure 2a

Effect of drug administration after the ATCC strain challenge

![Graph showing the effect of drug administration after the ATCC strain challenge.](image)

Figure 2b

Effect of drug administration after the multiresistant strain challenge

![Graph showing the effect of drug administration after the multiresistant strain challenge.](image)
Figure 3a

Effect of drug administration after the ATCC strain challenge

Time (hours)

Endotoxin Plasma Level (EU/ml)

Control
Tachyplesin III
Colistin
Imipenem
Tachyplesin III and Imipenem
Colistin and Imipenem

Figure 3b

Effect of drug administration after multiresistant strain challenge

Time (hours)

Endotoxin Plasma Level (EU/ml)

Control
Tachyplesin III
Colistin
Imipenem
Tachyplesin III and Imipenem
Colistin and Imipenem