

**Efficacy of the combination of tachypleisin III and clarithromycin in rat models of  
*Escherichia coli* sepsis**

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**Running Head: Tachypleisin III and clarithromycin in bacteraemia**

## Abstract

We investigated the efficacy of tachyplesin III and clarithromycin in two experimental rat models of Gram-negative severe infection.

Adult male Wistar rats were given i) an intraperitoneal injection of 1 mg/Kg *Escherichia coli* 0111:B4 LPS, ii)  $2 \times 10^{10}$  CFU of *Escherichia coli* ATCC 25922. For each model, animals received intraperitoneally isotonic sodium chloride solution, 1 mg/Kg tachyplesin III, 50 mg/Kg clarithromycin, and 1 mg/Kg tachyplesin III combined with 50 mg/Kg clarithromycin, respectively. Lethality, bacterial growth in blood and peritoneum, endotoxin and TNF-alpha concentrations in plasma were evaluated. All compounds reduced the lethality when compared to controls. Tachyplesin III exerted a strong antimicrobial activity and achieved a significant reduction of plasma endotoxin and TNF-alpha concentration when compared with control and clarithromycin-treated groups. Clarithromycin exhibited no antimicrobial activity but a good impact on endotoxin and TNF-alpha plasma concentrations. Combination between tachyplesin III and clarithromycin resulted in significant reduction in bacterial count proved to be the most effective treatment in reducing all variables measured.

Keywords: tachyplesin III, macrolides, sepsis, animal models

Gram-negative have often been implicated in the pathogenesis of severe infection, sepsis, and sepsis-related organ injury, which are important causes of death in critically ill patients (1,8,23). The presence of outer membrane provides an effective protective barrier in these organisms to antibiotics that might otherwise be active. Several studies have showed that this protective barrier, formed by a divalent cation-cross-linked matrix of LPS molecules on the outer leaflet of the outer membrane, can be breached via displacement of LPS-bound metals by antimicrobial peptides of diverse structural classes (28,29). Antimicrobial peptides are multifunctional as effectors of innate immunity on skin and mucosal surfaces and have demonstrated direct antimicrobial activity against various bacteria, viruses, fungi, and parasites. Particularly, antimicrobial peptides revealed immunomodulatory functions, including endotoxin-binding and -neutralizing ability, chemotactic activities, induction of cytokines and chemokines, promotion of wound healing, and angiogenesis (5,11,14-16,27). Tachyplesins are a group of antimicrobial peptides isolated from horseshoe crabs. Tachyplesin III (KWCFRVCYRGICYRKCR-NH<sub>2</sub>) isolated from Southeast Asian horseshoe crabs *Tachyplesus gigas* and *Carcinoscorpius rotundicauda*, consisting of 17 amino acids with two disulfide bridges is a representative antimicrobial peptide with cyclic  $\beta$ -sheet (17,24). It is exhibit a broad-spectrum activity against Gram-negative and -positive bacteria, fungi and even enveloped viruses at low concentrations (17,24). "New" macrolides such as clarithromycin, for the treatment of patients with various infectious diseases, were not clinically introduced until 40 years later (3). Recent reports have shown its strong anti-inflammatory properties in experimental study of Gram-negative sepsis (12,13).

Such active but not penetrating antibiotics may gain clinical use when combined with membrane active compounds able to increase the permeability of the outer membrane and thus, render Gram-negative susceptible to several hydrophobic antibiotics. The present experimental study aimed to investigate the in vitro interaction and the in vivo efficacy of a membrane active compound as tachyplesin III and a hydrophobic antibiotic as clarithromycin in two rat models of *E. coli* infections: the first (intraperitoneal administration of LPS) to evaluate the anti-endotoxin activity and the immunomodulatory effect of the compounds, the latter (*E. coli* induced peritonitis) to evaluate their antimicrobial activity and their impact on survival.

## Materials and Methods

### *Drugs*

Tachyplesin III (accession number P18252), molecular weight 2235.76 Da) was synthesized by 9-fluorenylmethoxycarbonyl (Fmoc) solid-phase chemistry (4,10). The protected peptidyl resin was treated with the mixture: 92% trifluoroacetic acid (TFA), 2% phenol, 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. Clarithromycin was obtained from Abbott S.p.A. Campoverde LT, Italy and dissolved in accordance with manufacturers' recommendations. Solutions were made fresh on the day of assay.

#### *Organisms and Reagents*

The commercially available quality control strain of *Escherichia coli* ATCC 25922 was used. For in vitro studies, we used five clinical isolates of *E. coli* cultured from hospitalized patients with infection admitted to the Ospedali Riuniti of Ancona, Italy. Endotoxin (*Escherichia coli* serotype 0111:B4; Sigma-Aldrich S.r.l., Milan, Italy) was prepared in sterile saline, aliquoted, and stored at -80°C for short periods.

#### *In vitro studies*

Laboratory standard powders were diluted in accordance with the recommendations suggested by manufacturers'. MICs were determined according to the procedures outlined by the Clinical and Laboratory Standards Institute (CLSI) (7). Experiments were performed in triplicate.

For synergy studies, ATCC control strain and five clinical strains of *E. coli* were tested by a checkerboard titration method. The fractionary inhibitory concentration (FIC) indexes were interpreted as follows: <0.5, synergy; 0.5-4.0, indifferent; and >4.0, antagonism (9). 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 colony counts at 24 h by the combination

compared to the most active single agent, and the number of surviving organisms in the presence of the combination had to be 100 cfu/ml below the starting inoculum.

Finally, the ability of tachyplesin III to permeabilize the membranes of the Gram-negative bacteria was determined as described by Ofek et al. (25). Briefly, a bacterial suspension (10  $\mu$ l;  $1 \times 10^5$  CFU) was inoculated onto microtiter plate wells containing 100  $\mu$ l of a serial twofold dilution (1,000 to 0.5  $\mu$ g/ml) of clarithromycin in Iso Sensitest-Broth (ISB) (Oxoid S.p.A., Milan). To each well, 10  $\mu$ l of test peptide 600 mg/l was added, to achieve a final test peptide concentration of 50  $\mu$ g/ml. The extent to which the MIC clarithromycin decreased between wells, in the presence or the absence of the test peptides, was calculated and was designated the peptide's permeabilizing activity.

#### *Animals*

A total of 120 adult male (3-5 months age range) Wistar rats weighting 300 to 400 g were used. All animals were housed in individual cages 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..

#### *Experimental Design*

Two experimental conditions were studied: i) intraperitoneal administration of LPS, ii) *E. coli* induced peritonitis.

i) four groups, each containing 15 animals, were anesthetized by an intramuscular injection of ketamine and xylazine (30 mg/Kg and 8 mg/Kg of body weight,

respectively) and injected intraperitoneally with 1.0 mg *E. coli* serotype 0111:B4 LPS in a total volume of 500 µl sterile saline. Immediately after injection, animals received intraperitoneally isotonic sodium chloride solution (control group C<sub>0</sub>), 1 mg/Kg tachyplesin III, 50 mg/Kg clarithromycin, and 1 mg/Kg tachyplesin III combined with 50 mg/Kg clarithromycin, respectively.

ii) *E. coli* ATCC 25922 was grown in brain-heart infusion broth. When bacteria were in the log phase of growth the suspension was centrifuged at 1000 g for 15 min, the supernatant was discarded, and the bacteria were resuspended and diluted into sterile saline. All animals (four groups, each containing 15 animals) were anesthetized as above mentioned. The abdomen of each animal was shaved and prepared with iodine. The rats received an intraperitoneal inoculum 1 ml saline containing 1×10<sup>9</sup> CFU of *E. coli* ATCC 25922. Immediately after bacterial challenge, animals received intraperitoneally isotonic sodium chloride solution (control group C<sub>1</sub>), 1 mg/Kg tachyplesin III, 50 mg/Kg clarithromycin, and 1 mg/Kg tachyplesin III combined with 50 mg/Kg clarithromycin, respectively.

Toxicity was evaluated on the basis of the presence of any drug related adverse effects, i.e. local signs of inflammation, anorexia, weight loss, diarrhea, fever, and behavioral alterations. In particular, to evaluate the physiological effects of tachyplesin III, temperature, pulse, blood pressure, respirations and oxygenation were monitored in a supplementary peptide-treated group without infection

#### *Evaluation of treatment*

On the basis of the kind of experiment, at the end of the study the rate of blood culture positivity, the quantities of bacteria in the intra-abdominal fluid, the rate of lethality, and

plasma endotoxin and TNF-alpha levels were evaluated. The animals were monitored for the subsequent 72 h.

In all models the presence of systemic symptoms was defined in analogy to the criteria applied for humans. Each animal was considered to be endotoxic or septic if it satisfied at least two of the following criteria: a) increased pulse rate; b) rectal temperature above 38° C or below 36°; c) increased breathing rate; and finally more than 12,000 or less 4,000 white blood cells per  $\mu\text{l}$  (21). The surviving animals were killed with 4% isoflurane, and blood samples for culture were obtained by aseptic percutaneous transthoracic cardiac puncture. In addition, to perform quantitative evaluations of the bacteria in the intraabdominal fluid, 10 ml of sterile saline was injected intraperitoneally, samples of the peritoneal lavage fluid were serially diluted, and a 0.1 ml volume of each dilution was spread onto blood agar plates. The limit of detection was  $\leq 2 \log_{10}$  CFU/ml. The plates were incubated both in air and under anaerobic conditions at 35°C for 48 h. The bacterial isolates were identified by biochemical assay.

For blood cultures (models ii) and determination of endotoxin and TNF-alpha in plasma (all models), 0.1 ml blood samples were collected from a tail vein 0, 2, 6, 12 and 36 h after injection of LPS or bacteria into a sterile syringe and transferred to tubes containing ethylenediaminetetraacetic acid tripotassium salt (EDTA-K<sub>3</sub>).

#### *Biochemical assays*

Endotoxin concentrations were measured by the commercially available *Limulus* amoebocyte lysate test (E-TOXATE<sup>®</sup>, Sigma-Aldrich). Plasma samples were serially two-fold diluted with sterile endotoxin-free water and were heat-treated for 5 min in a

water bath at 75°C to destroy inhibitors that can interfere with the activation. The endotoxin content was determined as described by the manufacturer. 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 test samples was calculated by comparison with the standard curve. TNF-alpha levels were measured using a solid phase sandwich enzyme-linked immunosorbent assay. The intensity of the color was measured in a MR 700 Microplate Reader (Dynatech Laboratories, Guernsey, United Kingdom) by reading the absorbance at 450 nm. The results for the samples were compared with the standard curve to determine the amount of TNF-alpha present. All samples were run in duplicate. The lower limit of sensitivity for TNF-alpha by this assay was 0.05 ng/mL..

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### *Statistical analysis*

Mortality rates between groups were compared by use of Fisher exact test. Qualitative results for blood cultures were analyzed by the  $\chi^2$  test (eventually corrected according to Yates method) or Fisher exact test, depending on the sample size. Quantitative evaluations of the bacteria in the intra-abdominal fluid cultures were presented as means  $\pm$  standard deviations (SDs); statistical comparisons between groups were made by analysis of variance. Post hoc comparisons were performed by Bonferroni's test. Plasma endotoxin and TNF- $\alpha$  mean values were compared between groups by non parametric analysis of variance (Kruskal-Wallis test, followed by standard procedure for multiple comparisons), due to the presence of censored data. Each comparison group contained 15 animals. Significance level was fixed at 0.05.

## **Results**

### *In vitro studies*

ATCC control strain was inhibited by tachyplesin III at concentration of 4 mg/l, while clarithromycin exhibited MIC of 128 mg/l, suggesting that it was not active against *E. coli*. In the synergy study, when the peptide was combined to the macrolide, we observed FIC index of 0.385. Time-kill synergy studies showed no effect when the compounds were tested at one-fourth the MIC. On the other hand, synergism was clearly observed at one-half the MIC: actually, the combination of the drugs determined at 24 h a decrease in colony count of 3 log ( $6.71 \pm 1.15 \times 10^3$ ) compared to tachyplesin III, the most active single agent, that produced at 24 h a colony count of  $5.65 \pm 1.31 \times 10^6$ .

Finally, the sensitivity of all strains to clarithromycin increased by up to four-fold in the presence of tachyplesin III (MIC range: 16-32 mg/l for the ATCC control strain, 8-32 mg/l for the five clinical isolates). In a separate set of experiments we determined the minimum concentration of the test peptide needed to render *E. coli* sensitive to 30 µg/ml of clarithromycin. We found that concentration of at least 2.0 mg/l of tachyplesin III was required to render the bacteria sensitive to clarithromycin.

#### *In vivo studies*

Model (i). Intraperitoneal administration of LPS.

Plasma peak levels of endotoxin and TNF- $\alpha$  were observed 6 h after intraperitoneal administration of 1.0 mg/Kg *Escherichia coli* serotype 0111:B4 LPS. Nevertheless, intravenous tachyplesin III treatments with or without clarithromycin resulted in marked decrease ( $P < 0.05$ ) of TNF- $\alpha$  and virtually undetectable levels of endotoxin in the plasma of tachyplesin III-treated groups, compared with those of control group ( $C_0$ ) and macrolide-treated group. Interestingly, significant differences in the plasma levels of both LPS and TNF- $\alpha$  were also observed between clarithromycin-treated and untreated groups (Figure 1).

Model (ii). Intraperitoneal injection of  $1 \times 10^9$  CFU of *E. coli* ATCC 25922.

All animals were monitored for 72 h. The rate of lethal ties in control group  $C_1$  was 100% within 48 h. All antibiotic treatments led to decreased mortality ( $P < 0.05$ ). In particular, a lethality rates of 33.3 and 66.6% were observed for group treated with tachyplesin III and clarithromycin, while a rate of 6.6% was observed in combined-

treated group (Table 1). In the same groups, bacteriological evaluation of C<sub>1</sub> showed 100% positive blood cultures, and  $7.3 \times 10^8 \pm 2.0 \times 10^8$  CFU/ml were counted in the intra-abdominal fluid. Tachyplesin III showed an higher antimicrobial activity than the macrolide. When it was combined with clarithromycin, the positive interaction produced the lowest bacterial counts ( $1.4 \times 10^1 \pm 0.2 \times 10^1$  CFU/ml) statistically significant versus all other groups ( $P < 0.05$ ). The administration of tachyplesin III alone or combined produced a strong reduction in plasma endotoxin and TNF-alpha levels compared to control and clarithromycin. However, clarithromycin showed a good anti-inflammatory activity with significant differences observed between this treated group and control. The results are summarized in Figures 2 and 3.

Finally, none of the tachyplesin III-treated animals had clinical evidence of drug-related adverse effects and no changes in physiological parameters were observed in the supplementary 1 mg/Kg peptide-treated group without infection.

## Discussion

The development of agents that can permeabilize the outer membranes of Gram-negative bacteria may provide a useful new approach to terminating the infectious process by enhancing the activity of antibiotics that might otherwise be inactive. These membranes act as an effective permeability barrier against external noxious agents, and lipopolysaccharide (LPS) is the key molecule for this function (28,29,31). In the outer membrane of Gram-negative bacteria, the LPS molecules occupy the outer leaflet of the membrane and leave no space for glycerophospholipids, which thus occupy the inner leaflet (32). Antimicrobial peptides are thought to play an important role in killing and

clearance of Gram-negative bacteria and the neutralization of endotoxin. Their target cell is the membrane and include the structure, length, and complexity of the hydrophilic polysaccharide found in outer layer (15). These parameters affect the ability of each peptide to diffuse through the cell's outer barrier and to reach its cytoplasmic plasma membrane. In order to exert their activity, antimicrobial peptides first interact with and traverse an outer barrier, mainly LPS and peptidoglycan in bacteria or a glycocalyx layer and matrix proteins in mammalian cells (26,30,33). Only then, can the peptides bind and insert into the cytoplasmic membrane. Recent studies have showed that the patterns of peptide-induced permeabilization of the outer and inner *E. coli* membranes correlated well with the antimicrobial activity, confirming that this permeabilization determine an harmful effect upon bacteria (19).

In the present study, we evaluated the efficacy of the combination between tachyplesin III and clarithromycin against two animal models of *E. coli* infection. In our models, the administration of peptide showed a good impact on lethality rates, plasma endotoxin and TNF-alpha levels. Its ability to inhibit the release of endotoxin and cytokines can explain the interesting finding concerning the in low TNF levels in the endotoxin group as well as the bacterial inoculated group. Clarithromycin exhibited a good anti-inflammatory activity, while its impact on lethality rates and bacteremia was significantly lower than tachyplesin III. It is important to note that the antibacterial activity of tachyplesin III was significantly increased when it was combined with clarithromycin, and this combination give statistically significant reduction on all outcome measures considered.

In time-killing curves and in checkerboard titration method, a strong synergistic effect was observed. This synergistic pattern clearly was also observed in the in vivo

setting. In fact, combination between tachyplesin III and clarithromycin resulted in significant decrease of bacterial count, positive blood cultures and lethality rates as compared with peptide monotherapy.

This combination was also most effective in decreasing the levels of LPS and TNF- $\alpha$ , confirming the good immunomodulatory activity of tachyplesin III and clarithromycin.

Previous studies have reported the positive interaction among antimicrobial peptides and hydrophobic antibiotics (6,33). Nevertheless, this mechanism is not known, with few exceptions. It is generally thought that antimicrobial peptides exert their inhibitory effects by increasing bacterial membrane permeability, causing leakage of bacterial contents. Membrane permeability-increasing agents by the loss of effective outer membrane porin channels, could greatly sensitizes Gram-negative organisms to otherwise impermeable hydrophobic solutes, facilitate the penetration and finally enhance its activity (2,22,33). Tachyplesins are also considered to exert its bactericidal activity by permeabilizing bacterial membranes, although the molecular mechanism has not yet been determined (18,20). Several studies have showed that they form an anion selective pore in planar lipid bilayer and triggers the leakage of calcein from liposomes, the latter being coupled to the translocation of the peptide across lipid bilayers. Tachyplesins form also a toroidal pore composed of peptides and lipids (18,20). These mechanisms of barrier-disturbing effect of tachyplesin III upon the outer membrane could be thereby provide the clarithromycin accessibility to its intra-cytoplasmatic target. It has been showed that macrolides use the hydrophobic pathway across the outer membrane and that the intact outer membrane is an effective barrier against them.

The antimicrobial and antiendotoxin activities of tachyplesin III and its synergistic interactions demonstrated upon clarithromycin highlight the potential usefulness of this combination in *E. coli* severe infections. More studies are needed to determine the safety and the efficacy of this antibiotic combination against severe Gram-negative infection.

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FIGURE 1 LEGEND PAGE

Endotoxin and TNF-alpha plasma levels after intraperitoneal administration of 1.0 mg *Escherichia coli* serotype 0111:B4 LPS

FIGURE 2 LEGEND PAGE

Effects on endotoxin plasma levels of 1 mg/Kg tachyplesin III, 50 mg/Kg clarithromycin and 1 mg/Kg tachyplesin III plus 50 mg/Kg clarithromycin administered intravenously after intraperitoneal injection of  $1 \times 10^9$  CFU of *E. coli* ATCC 25922

FIGURE 3 LEGEND PAGE

Effects on TNF-alpha plasma levels of 1 mg/Kg tachyplesin III, 50 mg/Kg clarithromycin and 1 mg/Kg tachyplesin III plus 50 mg/Kg clarithromycin administered intravenously at 0 and 360 min after intraperitoneal injection of  $1 \times 10^9$  CFU of *E. coli* ATCC 25922

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Figure 1a

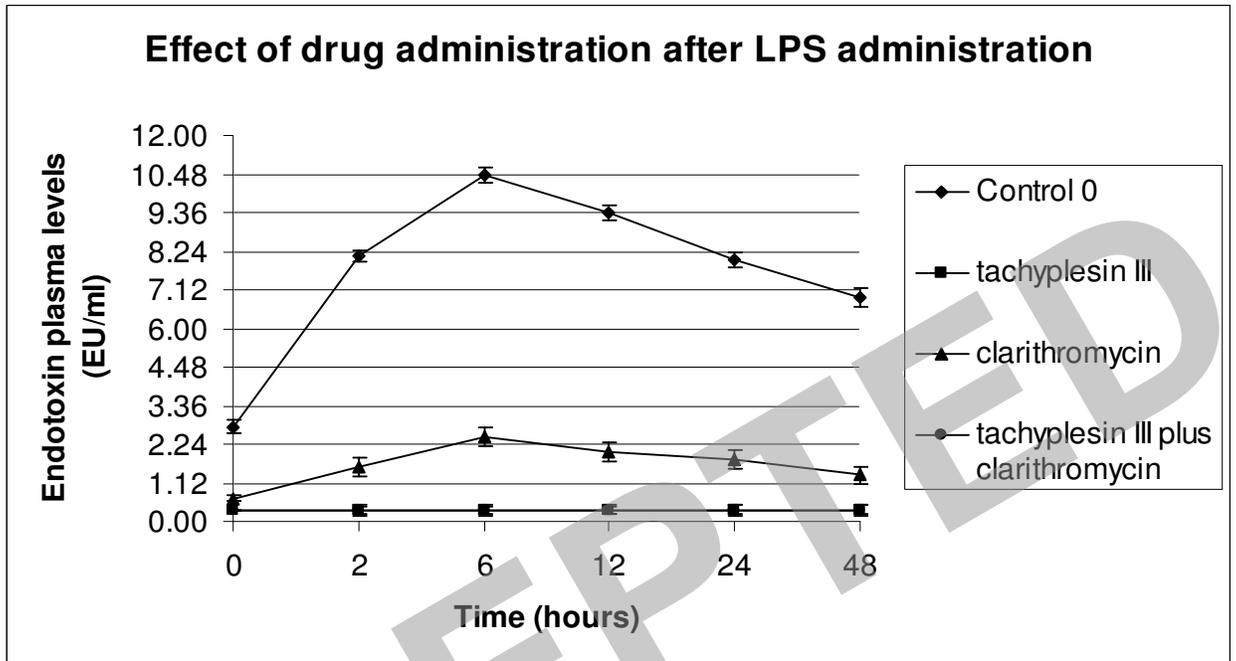


Figure 1b

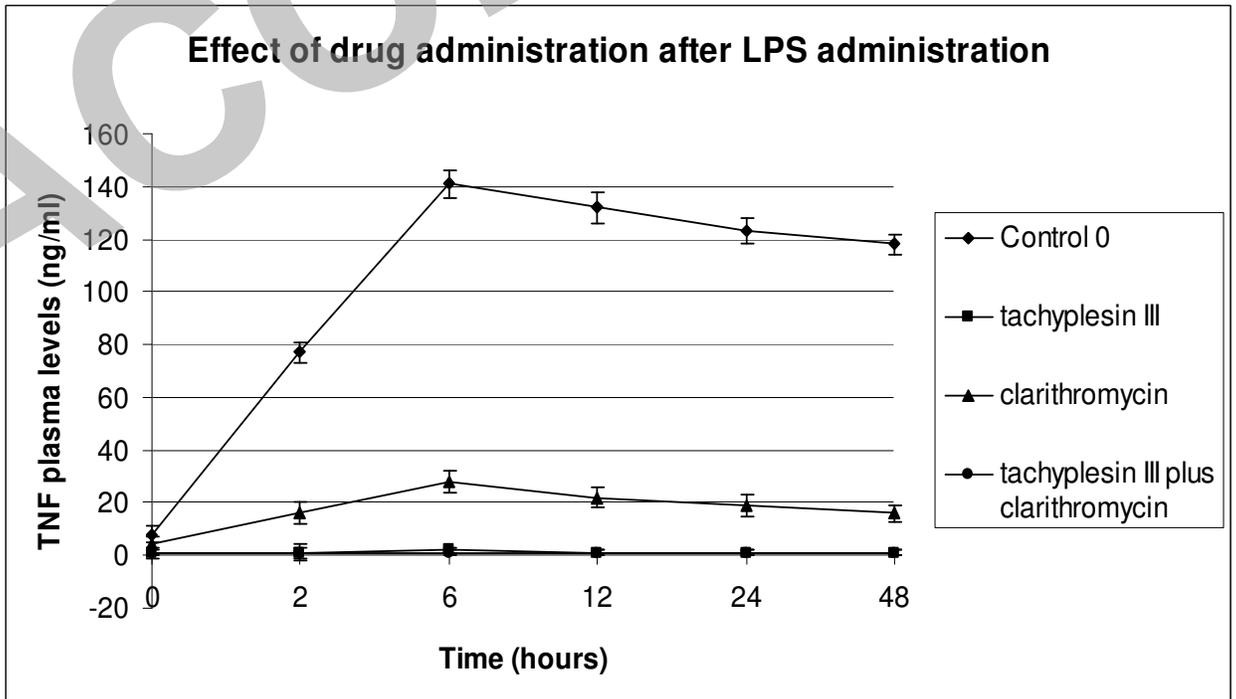


Figure 2

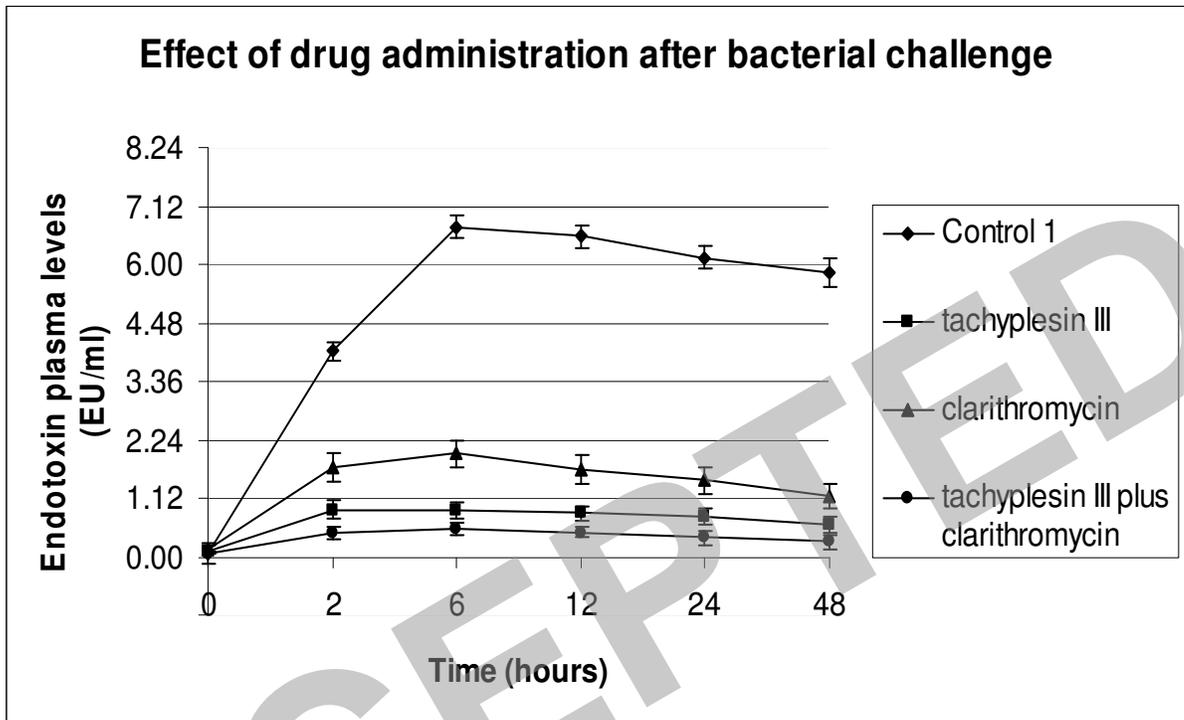


Figure 3

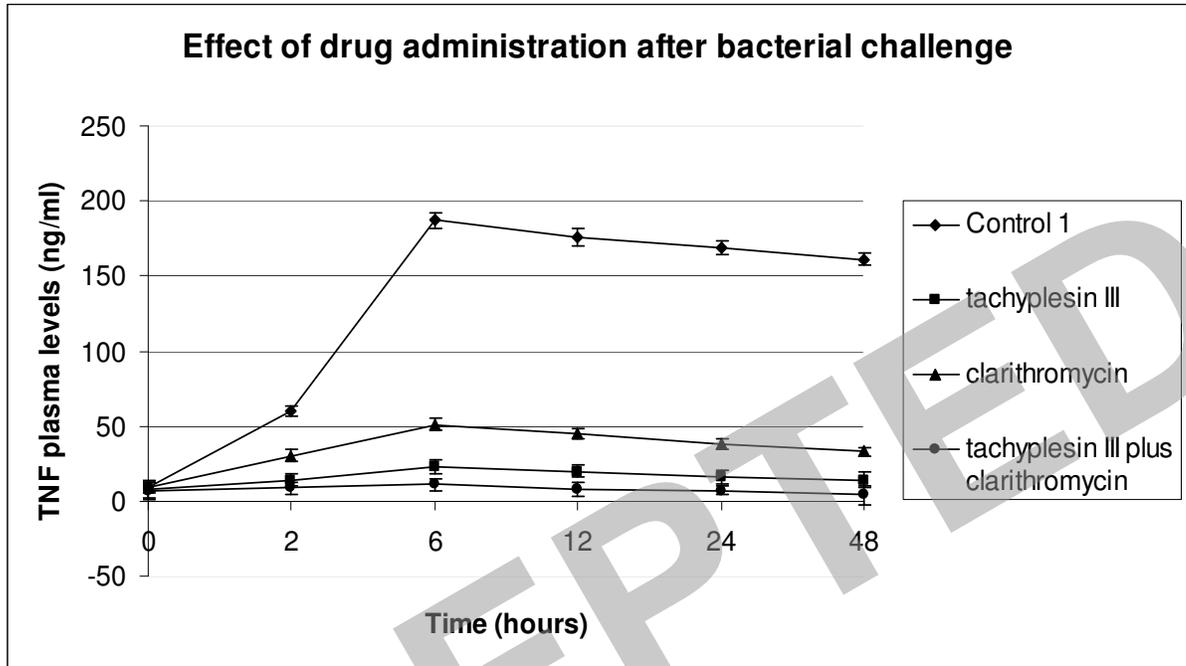


Table 1. Efficacy of administration of intravenous tachyplesin III and clarithromycin in a rat model after intraperitoneal injection of  $1 \times 10^9$  CFU of *E. coli* ATCC 25922

Treatment <sup>a</sup>	Lethality <sup>b</sup>		Qualitative blood culture	Bacterial count in peritoneal fluid (CFU/ml) <sup>c</sup>
	No. dead/total	%	No. positive/total	log
No treatment (control group)	15/15	100	15/15	8.86 ± 8,30
T-III mg/Kg	5/15 <sup>de</sup>	33.3	5/15 <sup>de</sup>	4.46 ± 3,84 <sup>de</sup>

CLR 50 mg/Kg	10/15 <sup>d</sup>	66.6	11/15 <sup>d</sup>	6.68 ± 6.04
T-III 1 mg/Kg plus CLR 50 mg/Kg	1/15 <sup>def</sup>	6.6	2/15 <sup>def</sup>	1.14 ± 0.30 <sup>def</sup>

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<sup>a</sup>T-III, tachyplesin III; CLR, clarithromycin.

<sup>b</sup>Lethality was monitored for 72 h following the challenge

<sup>c</sup>Mean ± S.D.

<sup>d</sup> $P < 0.05$  versus the control group C<sub>2</sub>

<sup>e</sup> $P < 0.05$  versus clarithromycin treated group

<sup>f</sup> $P < 0.05$  versus clarithromycin-treated group