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Temporin A Soaking in Combination with Intraperitoneal Linezolid Prevents Vascular Graft Infection in a Subcutaneous Rat Pouch Model of Infection with *Staphylococcus epidermidis* with Intermediate Resistance to Glycopeptides

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The efficacy of linezolid and temporin A in the prevention of prosthetic graft infection due to methicillin-resistant *Staphylococcus epidermidis* with intermediate resistance to glycopeptides was investigated in a subcutaneous rat pouch model. Linezolid and temporin A, alone or combined, greatly reduced the bacterial numbers compared to the effect with control drugs.

In recent years the effectiveness of new antimicrobial compounds to treat and prevent vascular graft infections has been evaluated in different experimental models (1, 3, 7, 14, 17). In fact, vascular graft infections remain a major surgical challenge, because prevention of risk factors and antibiotic therapy can reduce but not eradicate them (4, 8). Moreover, the continued isolation of methicillin-resistant staphylococci and the increase in the numbers of infections caused by the emergence of glycopeptide-resistant cocci among nosocomial strains have prompted the development of compounds specifically directed at the treatment of these pathogens (9, 13, 15, 16).

Few new agents have demonstrated significant in vitro activities against antibiotic-resistant staphylococci. One of these compounds is the oxazolidinone linezolid. Oxazolidinones are a new class of antimicrobials with a unique mechanism of action. They inhibit bacterial protein synthesis by binding to the 50S ribosomal subunit; this binding prevents formation of a functional initiation complex in bacterial translation systems. Linezolid has been approved by the Food and Drug Administration for use in treating infections caused by gram-positive organisms, including multidrug-resistant isolates of staphylococci, streptococci, and enterococci (5, 12).

Temporins are a family of linear 10- to 13-residue-long peptides with a net positive charge and an amidated C-terminal antimicrobial peptide. Initially, they were isolated from the skin of the European red frog, *Rana temporaria* (17, 18). They showed activity against clinically important gram-positive cocci, including multidrug-resistant staphylococci and vancomycin-resistant *Enterococcus faecium* (3, 17, 18). Temporin A is a basic, highly hydrophobic, antimicrobial peptide amide (FLPLIGRVLSG IL-NH^2) that, like the other temporins, is active against clinically important antibiotic-resistant gram-positive cocci (17–19).

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In this study we used one strain of *Staphylococcus epidermidis* with intermediate resistance to glycopeptides (GISE) to investigate the in vitro activities of temporin A and linezolid and their in vivo efficacies in preventing prosthesis infection in a rat model.

Organisms. A clinical isolate of methicillin-resistant *S. epidermidis* with intermediate resistance to glycopeptides (GISE), obtained from a hospitalized patient with a surgical wound infection, was studied. *S. epidermidis* ATCC 12228 was used as a quality control strain in the in vitro investigations.

Drugs. Temporin A was synthesized manually by the solid-phase method with the Fmoc (9-fluorenylmethoxy carbonyl)-Bu^t procedure (Faculty of Pharmacy, Medical University of Gdańsk, Gdańsk, Poland). Linezolid was obtained from Pharmacia & Upjohn, Kalamazoo, Mich. Vancomycin was obtained from Sigma-Aldrich, Milan, Italy. Teicoplanin was obtained from Aventis Pharma, Milan, Italy. Powders were diluted in accordance with manufacturers' recommendations.

Soaking Dacron in temporin A solution. The amount of temporin A that soaked into the Dacron was estimated using UV spectroscopy. First, a 1-cm^2 collagen-sealed Dacron graft (Albograft; Sorin Biomedica Cardio, S.p.A., Saluggia [VC], Italy) was washed with distilled water for 10 min. Afterwards Dacron was allowed to be soaked with Fmoc-temporin A (10 mg/liter) for 20 min at room temperature. The absorption spectrum of the solution of Fmoc-temporin A was measured at λ 266 nm with a UV–visible-light spectrometer (Lambda 40P; Perkin-Elmer, Norwalk, Conn.) before soaking. Immediately after soaking Dacron was taken out of the solution, and the absorption spectrum of the solution of Fmoc-temporin A was measured again. The amount of Fmoc-temporin A soaked into the Dacron was estimated based on differences in absorption before and after the above-mentioned procedure.

Antimicrobial susceptibility testing. Antimicrobial susceptibilities were determined by broth microdilution as described by the National Committee for Clinical Laboratory Standards

(NCCLS) (10). In addition, the isolates were tested for susceptibility to vancomycin by the NCCLS reference disk diffusion method with 30-μg vancomycin and teicoplanin disks (11). The experiments were performed in triplicate.

Synergy studies. In interaction studies, the strains were used to test the antibiotic combinations by a checkerboard titration method with 96-well polypropylene microtiter plates. The ranges of drug dilutions used were 0.125 to 64 mg/liter for temporin A and 0.25 to 256 mg/liter for clinically used antibiotics. The fractionary inhibitory concentration (FIC) index for combinations of two antimicrobials was calculated according to the equation FIC index = $FIC_A + FIC_B = A/MIC_A + B/MIC_B$, where A and B are the MICs of drug A and drug B in the combination, MIC_A and MIC_B are the MICs of drug A and drug B alone, and FIC_A and FIC_B are the FICs of drug A and drug B. The FIC indexes were interpreted as follows: <0.5, synergy; 0.5 to 4.0, indifference; and >4.0, antagonism (6).

Animals. Adult male Wistar rats (weight range, 260 to 330 g) were used for all the experiments. The study was approved by the Animal Research Ethics Committee of the I.N.R.C.A. I.R.R.C.S., University of Ancona, Ancona, Italy.

Rat model. The in vivo study included a control group; one contaminated group that did not receive any antibiotic prophylaxis; one contaminated group that received a temporin A-soaked graft; three contaminated groups that received intraperitoneally 8 mg of linezolid, 7 mg of vancomycin, and 3 mg of teicoplanin/kg of body weight; and three contaminated groups that received a temporin A-soaked graft and intraperitoneal linezolid, vancomycin, and teicoplanin at the above-mentioned concentrations. Each group included 10 animals, and to verify the results, the experiments were performed in duplicate. In the statistical analysis, the data were merged and referred to all 20 animals from each pair of groups. Rats were anesthetized with ether, the hair on the back was shaved, and the skin was cleansed with 10% povidone-iodine solution. The groups received, immediately before intervention and successively at established times, an intraperitoneal dose of linezolid or teicoplanin (every 12 h) or vancomycin (every 6 h) at the above-mentioned dosages for 7 days. One subcutaneous pocket was made on each side of the median line by a 1.5-cm incision. Aseptically, 1-cm^2 sterile collagen-sealed Dacron grafts (Albograft) were implanted into the pockets. Before implantation, the Dacron graft segments were soaked with 10 mg of temporin A/liter. Soaking of the compound was obtained immediately before implantation by immersion of grafts for 20 min in a sterile solution of the above-mentioned agent. The pockets were closed by means of skin clips, and a saline solution (1 ml) containing 2×10^7 CFU of GISE/ml was inoculated onto the graft surface by using a tuberculin syringe to create a subcutaneous fluid-filled pocket (2). The animals were returned to individual cages and thoroughly examined daily. Based on experiments demonstrating peak bacterial growth and biofilm formation within 72 h (data not shown), all grafts were explanted at 7 days following implantation. Experiments were performed in duplicate. Toxicity was evaluated on the basis of the presence of any drug-related adverse effects, i.e., local signs of perigraft inflammation, anorexia, weight loss, vomiting, diarrhea, fever, and behavioral alterations.

Assessment of the infection. The explanted grafts were placed in sterile tubes, washed in sterile saline solution, placed in tubes containing 10 ml of phosphate-buffered saline solution, and sonicated for 2 min to remove the adherent bacteria from the grafts. Quantification of viable bacteria was performed by culturing serial 10-fold dilutions (0.1 ml) of the bacterial suspension on blood agar plates. All plates were incubated at 37°C for 48 h and evaluated for the presence of the GISE strain. The organisms were quantified by counting the number of CFU per plate. The limit of detection for this method was approximately 10 CFU/ml.

Statistical analysis. MICs are presented as the modes of three separate experiments. All in vivo data were merged and referred to all 20 animals from each pair of groups. Quantitative culture results regarding the in vivo experiments are presented as means ± standard deviations of the means; for results below the lower limit of detection the value considered was 10 CFU. Data were analyzed by one-way analysis of variance; post-hoc multiple comparisons were performed by applying Bonferroni's criterion. Significance was accepted when the *P* value was equal to or less than the Bonferroni critical value 0.001786 (0.05/28, where 28 is the number of the pairs of compared groups).

Soaking Dacron in temporin A solution. The experiments showed that, when 1 cm^2 of Dacron was soaked in a solution of 10 mg of temporin A/liter, 37 μg of temporin A remained on the Dacron.

In vitro susceptibility studies. According to the broth microdilution method recommended by the NCCLS, vancomycin exhibited MICs of 0.25 and 8 μg/ml for *S. epidermidis* ATCC 12228 and GISE, respectively, while teicoplanin exhibited MICs of 0.25 and 16 mg/liter, respectively. The two strains were similarly susceptible to linezolid, which showed MICs of 1.00 and 2.00 μg/ml for *S. epidermidis* ATCC 12228 and the GISE strain, respectively. Finally, temporin A showed MICs of 2 μg/ml for both strains. The differential pattern of susceptibility was confirmed by the disk diffusion test: *S. epidermidis* ATCC 12228 showed zone sizes of 15 and 18 mm for teicoplanin and vancomycin, respectively, while the intermediate resistance of the GISE strain to the glycopeptides was demonstrated by zone sizes of 11 mm for both vancomycin and teicoplanin. In the combination studies synergy was never observed, with the exception of the combinations between temporin A and linezolid. Actually, the strains GISE and ATCC 12228 produced FIC indexes of 0.312 and 0.187, respectively, when temporin A was combined with linezolid, while the other experiments with vancomycin and teicoplanin gave values between 0.750 and 2.0 (data not shown).

In vivo studies. None of the animals included in the uncontaminated control group had microbiological evidence of graft infection. In contrast, all 20 rats included in the untreated control group demonstrated evidence of graft infection, with quantitative culture results showing $6.9 \times 10^6 \pm 2.1 \times 10^6$ CFU/ml. Rats that received linezolid showed the lowest bacterial numbers ($3.8 \times 10^2 \pm 0.9 \times 10^2$ CFU/ml). Temporin A showed also a good activity with bacterial numbers of $3.4 \times 10^3 \pm 7.9 \times 10^2$ CFU/ml. In contrast, for rats that received teicoplanin or vancomycin the quantitative graft cultures demonstrated $8.2 \times 10^4 \pm 1.5 \times 10^4$ or $6.8 \times 10^4 \pm 1.3 \times 10^4$ CFU/ml, respectively. All combinations showed efficacies higher than that of each single compound. In fact temporin A plus vancomycin or teicoplanin showed bacterial numbers of

TABLE 1. Activities of temporin A, linezolid, vancomycin, and teicoplanin against glycopeptide-intermediately resistant *S. epidermidis* in a rat model

Group	Drug for soaking graft[a]	Intraperitoneal preoperative drug[b]	Quantitative graft culture (CFU/cm^2)[c]
Uncontaminated control			<10
Untreated control GISE			$6.9 \times 10^6 \pm 2.1 \times 10^6$
GISE1[d]		Linezolid	$3.8 \times 10^2 \pm 0.9 \times 10^2$
GISE2[d]		Vancomycin	$6.8 \times 10^4 \pm 1.3 \times 10^4$
GISE3[d]		Teicoplanin	$8.2 \times 10^4 \pm 1.5 \times 10^4$
GISE4[d]	TemporinA		$3.4 \times 10^3 \pm 7.9 \times 10^2$
GISE5[d,e]	TemporinA	Linezolid	<10
GISE6[d]	TemporinA	Vancomycin	$6.0 \times 10^2 \pm 2.0 \times 10^2$
GISE7[d]	TemporinA	Teicoplanin	$5.3 \times 10^2 \pm 1.8 \times 10^2$

[a] The Dacron graft segments were soaked with 10 mg of temporin A/liter.
[b] Each rat received intraperitoneally 8 mg of linezolid, 7 mg of vancomycin, or 3 mg of teicoplanin per kg.
[c] Mean number ± standard deviation. The limit of detection for the method was ≤10 CFU/ml.
[d] Statistically significant compared with the untreated control group GISE result.
[e] Statistically significant compared with all groups.

10^2 CFU/ml per graft while the combinations between temporin A and linezolid exerted the strongest antistaphylococcal efficacies (Table 1). Overall, all comparisons showed significant differences ($P < 0.0001$), except for vancomycin versus teicoplanin ($P = 0.0031$), linezolid versus temporin A plus teicoplanin ($P = 0.0019$), and temporin A plus vancomycin versus temporin A plus teicoplanin ($P = 0.2519$). None of the animals included in any group died or had clinical evidence of drug-related adverse effects, such as local signs of perigraft inflammation, anorexia, vomiting, diarrhea, and behavioral alterations.

The in vitro results of this study show that temporin A, linezolid, vancomycin, and teicoplanin had similar activities against the control strain *S. epidermidis* ATCC 12228 while only temporin A and linezolid exhibited high activity against the GISE clinical strain.

The in vivo results confirmed the strong antistaphylococcal activity of temporin A and linezolid. In fact, statistically significant differences were observed between the groups that received topical temporin A or intraperitoneal linezolid treatment and those that received vancomycin or teicoplanin treatment, though it is important that no compound was able to inhibit completely the growth of the resistant strains when administered alone.

Based on the observations in the present study concerning the high in vitro activity and the prophylactic in vivo efficacy shown against a staphylococcal strain with decreased susceptibility to the glycopeptides, temporin A and linezolid appear to be promising compounds for preventing multidrug-resistant staphylococcal infections. In particular, the administration of a topical peptide such as temporin A combined with a parenteral antibiotic with a strong antistaphylococcal activity may become an important future consideration for chemoprophylaxis in vascular surgery.

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