Prophylactic Efficacy of Topical Temporin A and RNAIII-Inhibiting Peptide in a Subcutaneous Rat Pouch Model of Graft Infection Attributable to Staphylococci With Intermediate Resistance to Glycopeptides

Oscar Cirioni, MD; Andrea Giacometti, PhD; Roberto Ghiselli, PhD; Giorgio Dell’Acqua, PhD; Yael Gov, PhD; Wojciech Kamysz, PhD; Jerzy Łukasiak, PhD; Federico Mocchegiani, MD; Fiorenza Orlando, PhD; Giuseppina D’Amato, MD; Naomi Balaban, PhD; Vittorio Saba, PhD; Giorgio Scalise, PhD

Background—Bacteria that adhere to implanted medical devices play an important role in industry and in modern medicine. Staphylococci are among the most common pathogens that cause biomaterial infections. Vascular prosthetic graft infection is one of the most feared complications that the vascular surgeon treats, frequently resulting in prolonged hospitalization, organ failure, amputation, and death. A rat model was used to investigate the topical efficacies of temporin A and the quorum-sensing inhibitor RNAIII-inhibiting protein (RIP) as prophylactic agents of vascular prosthetic graft infections caused by *Staphylococcus aureus* and *Staphylococcus epidermidis* with intermediate resistance to glycopeptides.

Methods and Results—Graft infections were established in the back subcutaneous tissue of adult male Wistar rats by implantation of Dacron prostheses 1 cm² followed by topical inoculation with 2 × 10⁷ colony-forming units of bacterial strains. The study included, for each staphylococcal strain, a control group (no graft contamination), a contaminated group that did not receive antibiotic prophylaxis, and 6 contaminated groups that received grafts soaked with temporin A, RIP, rifampin, temporin A plus RIP, RIP plus rifampin, or temporin A plus RIP. The infection was evaluated by quantitative agar culture. When tested alone, temporin A and RIP showed comparable efficacies, and their efficacies were significantly higher than that of rifampin against both strains. All combinations showed efficacies significantly higher than that of each single compound. The combinations of temporin A and RIP exerted the strongest antistaphylococcal efficacies, eliminating infection by 100%.

Conclusions—The results of the present study make these molecules potentially useful for antimicrobial chemoprophylaxis in vascular surgery. *(Circulation. 2003;108:767-771.)*

Key Words: vasculature grafting infection peptides
Moreover, development of bacterial resistance to antibiotics such as methicillin and vancomycin additionally limits presently available therapeutic approaches.11–15

Final outcome often results in longer hospitalization, need of surgery with removal of the device, and even death.10,16 More recent strategies to overcome these problems have mainly focused on precoating of devices with antibiotics. In the case of vascular grafts, antimicrobials such as rifampin, bounded in high concentrations to prosthetic grafts, have been proposed as adjunctive prophylaxis.17,18 Today, novel associations of promising molecules with antimicrobial capacity are being tested.19

A novel way to prevent biofilm formation would be to interfere with bacterial cell-cell communication that leads to the virulence phenotype.20 The organization of the biofilm into complex structures is regulated by the exchange of chemical signals between cells in a process known as quorum sensing.

RNAIII inhibiting peptide (RIP) is a heptapeptide originally isolated from culture supernatants of S. xylosus and has strong activity against adhesion and virulence of S. aureus and S. epidermidis. This was shown both in vitro on cells and plastics21 and in vivo against planktonic bacteria22,23 and biofilms on Dacron grafts when other drug combinations and different bacterial strains were used.24 Its mechanism of action is different from common antibiotics, because instead of killing the bacteria, it inhibits cell-cell communication, leading to prevention of their adhesion and virulence. RIP competes with an effector quorum-sensing molecule RNAIII-activating peptide (RAP) and thus inhibits the phosphorylation of TRAP, which is the RAP target molecule. This action leads to reduced bacterial adhesion and inhibition of the downstream production of the regulatory RNA molecule termed RNAIII, which is responsible for toxin synthesis.20,22,25

An alternative to the use of commonly used antibiotics are temporins, which are antibacterial cationic peptides. Amphibian skin has proven to be an especially rich source of these isolates were described using the acronyms VISE (vancomycin-intermediate S. epidermidis) and VISA (vancomycin-intermediate S. aureus).

Methods

Organisms
The strains of S. epidermidis and S. aureus with intermediate resistance to vancomycin were isolated from clinical specimens submitted for routine bacteriological investigation to the Institute of Infectious Diseases and Public Health, University of Ancona, Italy. These isolates were described using the acronyms VISE (vancomycin-intermediate S. epidermidis) and VISA (vancomycin-intermediate S. aureus).

Drugs
Vancomycin and rifampin were obtained from Sigma-Aldrich S.r.l. Powders were dissolved in accordance with the manufacturer’s recommendations. Solutions were made fresh on the day of assay or stored at −80°C in the dark for short periods. The concentration range assayed for each antibiotic was 0.25 to 250 μg/mL.

Synthetic Peptides
Temporin A was synthesized manually by the solid-phase method using Fmoc/But procedure (Faculty of Pharmacy, Medical University of Gdansk) and was purified by reverse-phase (Vydac C-18, 10×250 mm) high-pressure liquid chromatography (HPLC) on a Knauer K501 2-pump system. The product was analyzed by HPLC, chemical analysis, matrix-assisted laser-desorption ionization mass spectrometry. Temporin A was dissolved in distilled H2O at 20 times the required maximal concentration. SUCCESSIVELY, serial dilutions of the peptide were prepared in 0.01% acetic acid containing 0.2% BSA in polypropylene tubes. The amide form of RIP (YSPWTNF-NH2) and FITC-labeled RIP [Cys(S, Fluorescein)-YSPWTNF-NH2] were synthesized by Neo-system as previously described and purified by HPLC to 99%.25 RIP was dissolved in distilled H2O at 20 times the required maximal concentration. Solutions of RIP and temporin A were made fresh on the day of assay or stored at −80°C in the dark for short periods.

Binding of RIP and Temporin A to Dacron
To determine how much RIP impregnates Dacron, FITC-RIP (10 mg/L) was applied to 1 cm2 sterile collagen-sealed Dacron graft (Albograft, Sorin Biomedica Cardio, S.p.A) for 20 minutes at room temperature. Fluorescence in unbound solution was determined at OD 485/530 nm in a Microplate Fluorescence Reader (FL 600, Bio-Tek) using KC4 software. The binding level of temporin A to Dacron was estimated using UV spectroscopy. Collagen-sealed Dacron graft 1 cm2 (Albograft, Sorin Biomedica Cardio, S.p.A.) was first washed with distilled water for 10 minutes. Next, Dacron was allowed to be impregnated by Fmoc-temporin A (10 mg/L) for 20 minutes at room temperature. Absorption spectra of Fmoc-temporin A was measured at A 266 nm with UV-vis spectrometer (Lambda 40P, Perkin-Elmer) either before or after impregnation.

Antimicrobial Susceptibility Testing
The antimicrobial susceptibilities of the strains to vancomycin, rifampin, RIP, and temporin A were determined by the broth microdilution method described by the National Committee for Clinical Laboratory Standards (NCCLS).33 The MIC was taken as the lowest antibiotic concentration at which observable growth was inhibited. In addition, the strains were tested for susceptibility to
vancomycin by the NCCLS reference disk diffusion method by using 30-μg vancomycin disks.\textsuperscript{34} Experiments were performed in triplicate.

**Rat Model**

Adult male Wistar rats (weight range, 250 to 300 g; Instituto Nazionale Riposo e Cura Anziani Instituto di Ricovero e Cura a Carattere Scientifico [INRCA IRRCS] animal facility, Ancona, Italy) were studied. The study included, for each staphylococcal strain, a control group (no graft contamination), a contaminated group that did not receive any antibiotic prophylaxis, and 6 treated groups (VISE1-6 and VISA1-6) that received grafts soaked with RIP, temporin A, rifampin, temporin A plus RIP, RIP plus rifampin, and temporin A plus RIP, respectively. Each group contained 15 animals. Rats were anesthetized with ether, the hair on the back was shaved, and the skin was cleansed with 10% povidone-iodine solution. One subcutaneous pocket was made on each side of the median line by a 1.5-cm incision. Aseptically, 1-cm² sterile collagen-sealed double velour knitted polyethylene terephthalate (Dacron) grafts (Albograft, Sorin Biomedica Cardio) were implanted into the pores. Before implantation, the Dacron graft segments were impregnated with 10 mg/L temporin A, RIP, and rifampin, alone or combined. Binding of the compounds was obtained immediately before implantation by soaking grafts for 30 minutes in a sterile solution. The animals were returned to individual cages and thoroughly examined daily. Based on experiments demonstrating peak bacterial growth before implantation by soaking grafts for 30 minutes in a sterile solution of the above-mentioned agent. The pockets were closed by means of skin clips, and physiological solution (1 mL) containing the bacterial suspension in 10 mmol/L of sodium HEPES buffer (pH 7.2) (Sigma-Aldrich) to minimize the carryover effect and sonicated for 5 minutes to remove the adherent bacteria. Quantitation of viable bacteria was obtained by performing serial dilutions (0.1 mL) of the bacterial suspension in 10 mmol/L of sodium HEPES buffer (pH 7.2) (Sigma-Aldrich) to minimize the carryover effect and by culturing each dilution on blood agar plates. All plates were incubated at 37°C for 48 hours and evaluated for the presence of the staphylococcal strains. The organisms were quantitated by counting the number of CFUs per plate. The limit of detection for this method was approximately 10 CFU/cm².

**Statistical Analysis**

MIC values are presented as the geometric mean of 3 separate experiments. Quantitative culture results from all groups are presented as mean±SD, and the statistical comparisons between groups were made using ANOVA on the log-transformed data with Tukey-Kramer honestly significant difference test. Significance was accepted when \( P \leq 0.05 \).

**Results**

**Binding of RIP and Temporin A to Dacron**

To determine how much RIP and temporin A bound to the Dacron graft, the graft was soaked in FITC-RIP or Fmoc-temporin A and absorbance was determined. These experiments show that when 1 cm² Dacron was soaked in 10 mg/L RIP or 10 mg/L temporin A solutions, 26 μg of RIP and 37 μg of temporin A bound to it.

**In Vitro Data**

According to the broth microdilution method recommended by the NCCLS, the isolate confirmed its intermediate resistance to vancomycin. Vancomycin exhibited MICs of 8 μg/mL and zone sizes of 11 mm by the disk diffusion test. Both Temporin A and rifampin showed MICs of 8 mg/L for both strains, whereas RIP did not demonstrate any in vitro activities against the 2 strains (MICs ≥128 mg/L), as expected considering its mechanism of action.

**In Vivo Data**

Our results indicate that none of the animals included in the negative control group (no graft contamination) had anatomic and microbiological evidence of graft infection. On the other hand, all 30 rats included in the contaminated controls (VISE and VISA) demonstrated evidence of graft infection, with quantitative culture results showing 4.3×10⁵±16.8×10⁵ CFU/cm² graft and 6.0×10⁶±2.4×10⁷ CFU/cm² graft, respectively, although there were no local signs of perigraft inflammation.

Among groups treated with a single agent, the RIP-treated groups (VISE1 and VISA1) showed the lowest bacterial growth 4.9×10⁴±1.1×10⁵ CFU/cm² graft and 7.5×10³±2.9×10⁵ CFU/cm² graft, respectively. Otherwise, the groups treated with local temporin A (VISE2 and VISA2) and local rifampin (VISE3 and VISA3) demonstrated bacterial growth of 5.3×10⁴±7.7×10⁵ CFU/cm² graft, 4.5×10⁴±8.1×10⁵ CFU/cm² graft, 5.0×10⁴±8.8×10⁵ CFU/cm² graft, and 7.3×10⁴±2.4×10⁵ CFU/cm² graft, respectively.

Finally, the RIP and temporin A combination showed the lowest bacterial growth with no evidence of staphylococcal infection and negative quantitative cultures for group VISE4 and 6.9×10⁴±1.3×10⁵ CFU/cm² graft for group VISA4, whereas the other combinations showed bacterial numbers within 10⁵ and 10⁶ CFU/cm² graft (Table).

In the results from quantitative bacterial graft cultures, there were always significant differences when the data obtained from treated groups were compared with those obtained from the untreated groups with the exception of rifampin-treated groups. Interestingly, for the S. epidermidis series, combination between RIP and temporin A was shown to be statistically significant compared with any other treatment (\( P \leq 0.05 \)). Similar statistical results were obtained in the S. aureus series. The results and the statistical comparisons between groups are summarized in the Table.

All agents did not show any toxicity, and none of the animals included in any group died or had clinical evidence of drug-related adverse effects.

**Discussion**

The difficulty in eradicating a vascular chronic infection related to microcolony and biofilm formation relies on the fact that bacteria in a biofilm are able to resist higher antibiotic concentrations than bacteria in suspension.\textsuperscript{35} Moreover, it was recently shown that biofilm formation can be considered a multicellular developmental process regulated by the exchange of chemical signals between cells.\textsuperscript{36} The recognition of the presence of a cell to cell communication


Efficacy of RIP, Temporin A, and Rifampin Against Staphylococcus epidermidis and Staphylococcus aureus With Intermediate Resistance to Glycopeptide Strains Causing Graft Infection in a Rat Model

<table>
<thead>
<tr>
<th>Group*</th>
<th>Graft-Bonded Drug†</th>
<th>Quantitative Graft Culture, CFU/cm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uncontaminated control</td>
<td>...</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Untreated control VISE</td>
<td>...</td>
<td>4.3×10⁶ ± 6.8×10⁶</td>
</tr>
<tr>
<td>VISE1‡</td>
<td>RIP</td>
<td>4.9×10⁶ ± 1.1×10⁶</td>
</tr>
<tr>
<td>VISE2‡</td>
<td>Temporin A</td>
<td>5.3×10⁶ ± 7.7×10⁶</td>
</tr>
<tr>
<td>VISE3</td>
<td>Rifampin</td>
<td>5.0×10⁶ ± 8.8×10⁶</td>
</tr>
<tr>
<td>VISE4‡§</td>
<td>RIP plus temporin A</td>
<td>&lt;10</td>
</tr>
<tr>
<td>VISE5‡</td>
<td>RIP plus rifampin</td>
<td>2.2×10² ± 0.8×10²</td>
</tr>
<tr>
<td>VISE6‡</td>
<td>Temporin A plus rifampin</td>
<td>8.9×10² ± 2.5×10²</td>
</tr>
<tr>
<td>Uncontaminated control</td>
<td>...</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Untreated control VISA</td>
<td>...</td>
<td>6.0×10² ± 2.4×10²</td>
</tr>
<tr>
<td>VISA1‡</td>
<td>RIP</td>
<td>7.5×10² ± 2.9×10³</td>
</tr>
<tr>
<td>VISA2‡</td>
<td>Temporin A</td>
<td>4.5×10⁴ ± 8.1×10¹</td>
</tr>
<tr>
<td>VISA3</td>
<td>Rifampin</td>
<td>7.3×10⁴ ± 2.4×10⁶</td>
</tr>
<tr>
<td>VISA4‡</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VISA5‡</td>
<td>RIP plus rifampin</td>
<td>5.6×10⁴ ± 1.7×10²</td>
</tr>
<tr>
<td>VISA6‡</td>
<td>Temporin A plus rifampin</td>
<td>3.2×10³ ± 1.1×10³</td>
</tr>
</tbody>
</table>

VISE indicates Staphylococcus epidermidis with intermediate resistance to glycopeptides; VISA, Staphylococcus aureus with intermediate resistance to glycopeptides.

§Statistically significant when compared with control groups VISE and VISA.
‡Statistically significant when compared with all VISA groups, with the exception of VISA5.
†Dacron graft segments were impregnated with 10 mg/L of RIP, temporin A, or rifampin.
*Each group was formed by 15 animals.

will allow new approaches for prevention and treatment of the persistent infections stemming from biofilm.

With the aim of determining the antibiotic efficacies on biofilm bacteria, studies were carried out involving in vivo formation of microcolonies and biofilms, but most or all the antibiotics used were only partially effective.6,8,10 Furthermore, the recent emergence of multidrug resistance in coagulase-negative staphylococci heightens concerns and need of new prophylactic and therapeutic strategies. Since the emergence of methicillin-resistant staphylococci, glycopeptides were often the only effective drugs. For this reason, the emergence of staphylococcal strains exhibiting reduced sensitivity to vancomycin is of particular concern.15,37,38

In these experiments we used clinical isolates of vancomycin-resistant strains because they represent the most recent pattern of resistance that can be observed in the clinical practice and are more challenging to cure with classical antibiotics.12

One way to overcome the problems of emergence of resistance is the use of new antimicrobial compounds or combination therapy. Although several agents have been subjected to chemical manipulations to yield increased resistance to enzymatic inactivation, antibiotic-resistant mutants have usually emerged quickly. Recently, polycationic pep-

tides, crucial components of the innate immunity of both invertebrates and vertebrates and conserved theme in host antimicrobial defenses, have been proposed as a new class of antimicrobial agents. Among these peptides and their potential role in stimulating the defense of the host, defensins59,40 and temporins have been suggested as a new alternative to classical antibiotics, and some of them are quickly moving toward clinical applications.41,42 Temporins are among the smallest antimicrobial peptides so far described. They are all amidated at the C-terminus; those containing one basic residue, either lysine or arginine, in the sequence (net charge +2) were found to be active against Gram-positive pathogens, specifically staphylococci.29,30 Previous studies showed the efficacy of temporin A to provide prophylaxis against methicillin-susceptible and methicillin-resistant S. epidermidis graft contamination. In fact, it was demonstrated that prophylaxis based on temporin A–soaked grafts was as effective as parenteral vancomycin.42

Other prophylactic and therapeutic strategies could be based on compounds such as RIP, able to interfere with cell to cell communication, required for biofilm formation, and able to reduce bacterial adherence and the contact area each bacterium makes with the plastic surface.20–25 RIP is a quorum-sensing inhibitor of staphylococci that can adsorb to surfaces and act against bacterial biofilm on several synthetic polymers. For these reasons, in this study both the antistaphylococcal activity of temporin A and the disruption of cell to cell communication by RIP absorbed onto Dacron graft were tested.

For testing, we used a topical treatment model for prophylaxis instead of a more common systemic treatment. We believe that treatment in situ if successful will limit the systemic side effect of drugs given parenterally and allow a lower dosage. Indeed, we show that although a limited amount of the drugs bind to the graft, efficacy is maintained. Although we believe that the drugs will eventually be washed off the graft, their binding and their efficacy should be long enough to prevent the initial bacterial infection that could arise mostly during the surgical procedure. Additional experiments will be needed to establish in vivo duration of drug binding and efficacy and protection from secondary infections.

Our data show that the use of temporin A–soaked and RIP-soaked Dacron graft can result in significant bacterial growth inhibition even if high concentrations of organisms are topically inoculated on the Dacron prostheses. In particular, the results of the present study, if compared with those obtained in our recent report, point out the high antistaphylococcal activity of temporin A independently of the level of resistance shown by the isolates.42 On the other hand, RIP was shown to be highly effective also when tested alone and was more effective against both staphylococcal strains than either temporin A or rifampin alone.

In conclusion, we have shown in this paper that the use of novel drugs that do not belong to the classic antibiotics but that are derived either from the host or the bacteria itself prove to be very effective and even superior to antibiotics in inhibiting biofilm formation induced by drug-resistant staphylococci. The administration of a parenteral peptide such as
temporin A and topical RIP may become an important future consideration for chemoprophylaxis in vascular surgery.

References

Prophylactic Efficacy of Topical Temporin A and RNAIII-Inhibiting Peptide in a Subcutaneous Rat Pouch Model of Graft Infection Attributable to Staphylococci With Intermediate Resistance to Glycopeptides

Oscar Cirioni, Andrea Giacometti, Roberto Ghiselli, Giorgio Dell'Acqua, Yael Gov, Wojciech Kamysz, Jerzy Lukasiak, Federico Mocchegiani, Fiorenza Orlando, Giuseppina D'Amato, Naomi Balaban, Vittorio Saba and Giorgio Scalise

Circulation. 2003;108:767-771; originally published online July 28, 2003; doi: 10.1161/01.CIR.0000083717.85060.16

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2003 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/108/6/767

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation is online at:
http://circ.ahajournals.org/subscriptions/