Comparative Analysis of the Bactericidal Activities of Amphibian Peptide Analogues against Multidrug-Resistant Nosocomial Bacterial Strains

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Due to the widespread resistance of bacteria to the available drugs, the discovery of new classes of antibiotics is urgently needed, and naturally occurring antimicrobial peptides (AMPs) are considered promising candidates for future therapeutic use. Amphibian skin is one of the richest sources of such AMPs. In the present study we compared the in vitro bactericidal activities of five AMPs from three different species of anurans against multidrug-resistant clinical isolates belonging to species often involved in nosocomial infections (Staphylococcus aureus, Enterococcus faecium, Pseudomonas aeruginosa, Stenotrophomonas maltophilia, and Acinetobacter baumannii). The peptides tested were temporins A, B, and G from Rana temporaria; the fragment from positions 1 to 18 of esculentin 1b [Esc(1-18)] from Rana esculenta; and bombinin H2 from Bombina variegata. When they were tested in buffer, all the peptides were bactericidal against all bacterial species tested (three strains of each species) at concentrations ranging from 0.5 to 48 μM, with only a few exceptions. The temporins were found to be more active against gram-positive bacteria, especially when they were assayed in human serum; Esc(1-18) showed fast and strong bactericidal activity, within 2 to 20 min, especially against the gram-negative species, which were killed by Esc(1-18) at concentrations ranging from 0.5 to 1 μM; bombinin H2 displayed similar bactericidal activity toward all isolates. Interestingly, while the activities of the temporins and bombinin H2 were almost completely inhibited in the presence of 20% human serum, the activity of Esc(1-18) against the gram-negative species was partially preserved in the presence of 40% serum. This property renders this peptide an attractive molecule for use in the development of new compounds for the treatment of infectious diseases.

In recent years, the emergence of pathogenic microorganisms that have acquired resistance to a wide range of formerly efficacious antibiotics has become a major cause of concern both in hospital settings and in the community (1, 12). In some cases, bacterial strains with acquired resistance to every drug currently available have been selected, creating the prospect for a return to the situation in the preantibiotic era for infections caused by such strains (38). In this context, the identification of molecules endowed with antimicrobial activities, new mechanisms of action, and therapeutic potential is urgently needed. Naturally occurring antimicrobial peptides (AMPs) are effector molecules of innate immunity produced by a variety of multicellular organisms (3, 10, 39). Hundreds of AMPs have already been isolated from different biological sources (see an updated list at http://www.bbcm.univ.trieste.it/~tossi/pag1.htm) or designed de novo and synthesized. Despite substantial variations in their chain lengths and structures, most AMPs bear a net positive charge and a potential to adopt amphipathic α-helix and/or β-sheet structures (i.e., structures with separate hydrophobic and hydrophilic faces) upon interaction with the phospholipid membrane of the target cell. These two attributes are crucial for their ability to permeate and damage a biological membrane in order to exert their activity (30, 31). The possible use of these molecules or their derivatives as a new class of antibiotics is increasingly being taken into consideration for a number of reasons (13, 28). Among these is the observation that the rate of acquisition of resistance to AMPs by a sensitive microbial strain is several orders of magnitude lower than that to conventional antibiotics (14, 39). Indeed, the main target of AMPs is the bacterial membrane, and it is generally agreed that changing the composition and/or the organization of its lipids would not be evolutionarily advantageous for a microbial species (27). The therapeutic use of several AMPs is currently under investigation in animal models, while a few peptides are being tested in clinical trials as topical or systemic anti-infective agents (13, 24).

The skin of amphibians, especially that of the frogs of the genus Rana, is a particularly rich source of AMPs, with each species producing its own specific set of peptides (7) which are stored in granules of holocrine-type dermal glands and which

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are released into skin secretions as a reaction to stress, injury, or contact with microorganisms (22, 26).

Temporins were initially isolated from the European red frog (Rana temporaria) (18, 33) and now make up the largest family of AMPs from amphibians (more than 50 members), with properties that make them promising molecules for use in the future design of new anti-infective agents (20). They are among the smallest amphipathic α-helical AMPs (10 to 16 amino acids) found in nature to date, with a low net positive charge (from 0 to +3) at neutral pH and with a broad spectrum of biological activities, such as antimicrobial functions, chemotactic effects on human phagocytes, and the ability to modulate secretory phospholipase A₂ activity (5, 18). In addition, according to their ability to bind in vitro lipopolysaccharide (LPS; or endotoxin), the major component of the outer membrane of gram-negative bacteria, it has recently been demonstrated that temporins, alone or in combination with conventional antibiotics, reduce lethality in animal models of bacterial sepsis (6, 9).

A large variety of AMPs has also been isolated from Rana esculenta skin secretions (32, 35). Among these, the esculentin-1 family includes 46-amino-acid-residue peptides that display the most potent antimicrobial activities with negligible toxic effects on eukaryotic cells (16). Analysis of the analogues of esculentin-1b has revealed that the antimicrobial properties of the peptide are confined to its N-terminal region from positions 1 to 18 [Esc(1-18)], which possesses the same net positive charge (+5) as the full-length peptide (16).

Two families of AMPs from the skin secretions of Bombina species have been described: the bombinins and the relatively hydrophobic bombinins H (net charge, +3). The latter family includes a number of 20-residue alpha-helical peptides containing a D-amino acid in the second position, in addition to their corresponding all-L isomers (17, 21, 25, 34).

Intense research focusing on AMPs from frog skin is currently devoted to the elucidation of their mechanisms of action. Less widely investigated is the spectrum of bactericidal activity exhibited by such peptides against clinically relevant bacterial strains both in buffer and, especially, under experimental conditions more closely related to the in vivo situation (e.g., in the presence of biological fluids).

The aim of the present study was to compare the bactericidal activities of five AMPs from three different species of anurans (Rana temporaria, Rana esculenta, and Bombina variegata) against different human clinical isolates. The peptides taken into account were (i) temporins A, B, and G [TA, TB, and TG, respectively] from Rana temporaria; (ii) Esc(1-18) from Rana esculenta; and (iii) bombinin H2 (H2) from Bombina variegata. The activities of the peptides against multidrug-resistant bacteria isolated from clinical samples belonging to five species (three strains each) were assayed. These species included microorganisms commonly involved in nosocomial infections (Staphylococcus aureus, Enterococcus faecium, and Pseudomonas aeruginosa), as well as emerging pathogens, such as Stenotrophomonas maltophilia and Acinetobacter baumannii, that represent growing causes of life-threatening infections in intensive care unit patients (8). Since the species mentioned above are often involved in bloodstream infections, we also tested the activities of the peptides in the presence of human serum, a complex biological fluid known to inhibit the AMPs of different origins (2, 37). Overall, the peptides displayed bactericidal activities against all the microorganisms tested, although with some differences in their potencies, kinetics of action, and ability to maintain their antibacterial properties in serum, depending on the bacterial species considered.

### MATERIALS AND METHODS

**Peptides.** The main characteristics of the peptides used in this study are summarized in Table 1. TA, TB, and TG were synthesized by the use of the standard N-(9-fluorenylmethoxy carbonyl (Fmoc) amino acid derivatives with a 5-(4-Fmoc-aminomethyl-3,5-dimethoxy-phenox) valeric acid polyethylene glycol resin on an automated peptide synthesizer (ABI 433A; Applied Biosystems Inc., Foster City, CA). Esc(1-18) and H2 were purchased from Epytop (Nîmes, France). The purities of the peptides were confirmed by high-performance liquid chromatography analysis, and their sequences were determined both by automated Edman degradation with a protein sequencer (model 120C; Applied Biosystems) and by mass spectral analysis with a matrix-assisted laser desorption ionization–time of flight Voyager DE instrument (Applied Biosystems). The concentrations of the peptides were determined by quantitative amino acid analysis after acid hydrolysis with a Beckman system Gold instrument equipped with an ion-exchange column and ninhydrin derivatization. All peptides were dissolved in 20% ethanol and stored at −20°C until future use.

**Bacterial strains.** Clinical samples were obtained from distinct body sites of patients undergoing solid-organ transplantation at the University Hospital of Pisa, Pisa, Italy. The bacterial strains were isolated at the Microbiology Unit of the same hospital by standard procedures. Identification and susceptibility testing of the bacterial strains were performed by using VITEK 2 automatic instruments (BioMerieux, Lyon, France). Multidrug-resistant isolates belonging to five different bacterial species (three strains each) were selected for the study: *Enterococcus faecium* strains 3, 5, and 6; *Staphylococcus aureus* strains 1, 2, and 3; *Stenotrophomonas maltophilia* strain 1, 2, and 4; *Acinetobacter baumannii* strains 1, 2, and 3; and *Pseudomonas aeruginosa* strains 1, 2, and 3. The isolates were considered multidrug resistant if they were resistant to at least three drugs from distinct classes of antibiotics. The drug susceptibilities of *S. maltophilia* and *A. baumannii* and the Vancomycin susceptibility of *E. faecium* were confirmed by using Etest (AB Biodisk, Solna, Sweden). The resistance profiles of the clinical isolates used in this study are depicted in Table 2. The bacterial strains were grown in tryptone soy broth (Oxoid, Basingstoke, United Kingdom) until mid-log phase, subdivided into aliquots, and kept frozen at −80°C until future use.

**Bactericidal activity in SPB.** The bactericidal activities of the peptides against all clinical isolates were evaluated by a liquid microdilution assay in 10 mM sodium phosphate buffer (SPB; pH 7.4), as described previously (15). Briefly, exponentially growing bacteria were resuspended in SPB to obtain a density of 1 × 10⁸ CFU/ml. Ten microliters of each bacterial suspension was incubated at 37°C for various times (from 2 min to 1.5 h) in the presence of different concentrations of each peptide in 100 μl of SPB. Following incubation, the samples were plated onto tryptone soy agar (Oxoid, Basingstoke, United Kingdom). The number of CFU was determined after 24 h of incubation at 37°C. Bacterial activity was defined as a reduction in the numbers of viable bacteria of ≥3 log₁₀ CFU/ml at any of the incubation times tested.

**Bactericidal activity in serum.** Blood was drawn from the antecubital veins of five healthy donors and spontaneously allowed to clot at room temperature. After centrifugation at 300 × g, the serum samples were mixed and heat inactivated at 56°C for 30 min. Serum was added to the bactericidal assay media at a final concentration of 20% or 40%.

### TABLE 1. Primary structures and related net charges, at neutral pH, of the antimicrobial peptides used in this study

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Amino acid sequence</th>
<th>Net charge</th>
<th>Length (no. of amino acids)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TA</td>
<td>FLPLIGVRVGLGIL-NH₂</td>
<td>+2</td>
<td>13</td>
</tr>
<tr>
<td>TB</td>
<td>LLIVGVNLKSSL-NH₂</td>
<td>+2</td>
<td>13</td>
</tr>
<tr>
<td>TG</td>
<td>FFPVGRILNGIL-NH₂</td>
<td>+2</td>
<td>13</td>
</tr>
<tr>
<td>Esc(1-18)</td>
<td>GIFSKLAGKKLNLISG-NH₂</td>
<td>+5</td>
<td>18</td>
</tr>
<tr>
<td>H2</td>
<td>IIGPVLGLVSAGLGLIKKI-NH₂</td>
<td>+3</td>
<td>20</td>
</tr>
</tbody>
</table>
RESULTS

Comparison of bactericidal activities of amphibian peptides against nosocomial multidrug-resistant strains in SPB and human serum. The antimicrobial properties of five amphibian peptide analogues against five bacterial species (three strains each) were evaluated and the bactericidal activities, as defined in Materials and Methods, after 1.5 h of incubation of the strains with the different peptides in SPB were determined. As shown in Table 3, in most of the cases the peptides were bactericidal against the bacterial species and strains tested at concentrations ranging from 0.5 to 48 μM. Higher bactericidal concentrations were observed only for T_A and T_G against strain 4 of S. maltophilia and for T_A against strain 3 of P. aeruginosa. Esc(1-18) was the most powerful peptide against all three species of gram-negative bacteria, being bactericidal at concentrations as low as 0.5 to 1 μM. The same peptide was also active against E. faecium at relatively low concentrations (2 μM), while it killed S. aureus but did so only at concentrations of 8 to 32 μM. H2 displayed bactericidal activities at concentrations ranging from 4 to 16 μM against both gram-positive and gram-negative bacteria.

Most of the peptides had similar activities against clinical isolates belonging to the same species, although only three strains of each species were tested. A relatively high degree of variability was observed only in the cases of TA, TB, TG, and H2 against the three strains of S. maltophilia. Interestingly, Esc(1-18), besides being the most powerful peptide against the latter species, also displayed the least interstrain variability.

<table>
<thead>
<tr>
<th>Organism group and species</th>
<th>Strain no.</th>
<th>Concen (μM) required for a bactericidal effect*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram-positive species</td>
<td></td>
<td>SPB</td>
</tr>
<tr>
<td>E. faecium</td>
<td>3</td>
<td>12 12 6 2 4</td>
</tr>
<tr>
<td>E. faecium</td>
<td>5</td>
<td>12 6 2 8</td>
</tr>
<tr>
<td>E. faecium</td>
<td>6</td>
<td>12 6 2 4</td>
</tr>
<tr>
<td>S. aureus</td>
<td>1</td>
<td>12 8 24 4</td>
</tr>
<tr>
<td>S. aureus</td>
<td>2</td>
<td>12 12 8</td>
</tr>
<tr>
<td>S. aureus</td>
<td>3</td>
<td>12 12 12</td>
</tr>
<tr>
<td>Gram-negative species</td>
<td></td>
<td>Human serum</td>
</tr>
<tr>
<td>S. maltophilia</td>
<td>1</td>
<td>12 48 24 12 0.5 4</td>
</tr>
<tr>
<td>S. maltophilia</td>
<td>2</td>
<td>12 3 3 0.5 4</td>
</tr>
<tr>
<td>S. maltophilia</td>
<td>4</td>
<td>96 48 &gt;96 0.5 16</td>
</tr>
<tr>
<td>A. baumannii</td>
<td>1</td>
<td>24 24 12 0.5 4</td>
</tr>
<tr>
<td>A. baumannii</td>
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<td>12 6 6 0.5 4</td>
</tr>
<tr>
<td>A. baumannii</td>
<td>3</td>
<td>12 6 6 1 8</td>
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<tr>
<td>P. aeruginosa</td>
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<td>48 48 12 16</td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>3</td>
<td>96 48 48 16</td>
</tr>
</tbody>
</table>

* AMC, amoxicillin; AMK, amikacin; AMP, ampicillin; CAZ, ceftazidime; CFZ, cefazolin; CIP, ciprofloxacin; CLI, clindamycin; CRO, ceftriaxone; CTX, cefotaxime; CXM, cefuroxime; ERY, erythromycin; FEP, cefepime; GEN, gentamicin; IPM, imipenem; KAN, kanamycin; LVX, levofloxacin; MEM, meropenem; OXA, oxacillin; PIP, piperacillin; RIF, rifampin; SAM, ampicillin-sulbactam; SXT, trimethoprim-sulfamethoxazole; TEC, teicoplanin; TET, tetracycline; TOB, tobramycin; TZP, piperacillin-tazobactam; VAN, vancomycin.
variability in its activity, being bactericidal at a concentration of 0.5 μM against all three clinical isolates of *S. maltophilia*.

In order to evaluate whether the peptides under investigation retained their bactericidal activities in the presence of a complex biological fluid, bactericidal assays were performed in the presence of 20% human serum with one strain of each bacterial species. As depicted in Table 3, all peptides showed bactericidal activities against the two strains of gram-positive species tested, although at concentrations 2- to 16-fold higher than those observed in SPB. In contrast, the bactericidal activities of the temporins and H2 against most of the strains of the three gram-negative species in the presence of serum were highly inhibited (bactericidal concentrations at least 192 μM). Interestingly, in 20% serum Esc(1-18) displayed bactericidal activity at relatively low concentrations, ranging from 4 to 16 μM, against all three strains of the gram-negative species tested (Table 3). In addition, the activity of this peptide against the gram-negative species *A. baumannii* and *S. maltophilia* was also partially preserved in the presence of 40% human serum (Table 3).

**Killing kinetics of amphibian peptides against gram-positive and gram-negative nosocomial strains.** The killing kinetics of the five amphibian peptides against one representative strain of each bacterial species, arbitrarily chosen from among those tested, at 1.5 h were determined (Fig. 1 and 2). In these experiments each peptide was used at concentrations equal to or twofold greater than those able to give a bactericidal effect after 1.5 h of incubation. Esc(1-18) at either the bactericidal concentration or the twofold greater concentration did not have a bactericidal effect against the two gram-positive species within 30 min of incubation (Fig. 1). In contrast, TA, TB, TG, and H2 displayed bactericidal activities within 5 to 20 min against *E. faecium* when they were tested at their bactericidal concentrations (Fig. 1A) and had very fast killing effects (within 2 min) when they were assayed at concentrations twofold greater than their bactericidal concentrations (Fig. 1B). Only TB and TG displayed fast (5 to 10 min) bactericidal effects against *S. aureus*, which occurred with twofold the bactericidal concentrations, while TA and H2 showed killing activities against *S. aureus*, but the activities did not occur earlier than 30 min (Fig. 1C and D).

All peptides demonstrated very rapid killing kinetics against *S. maltophilia*; at twofold the bactericidal concentrations they killed the strain of this bacterial species within 2 to 5 min of incubation (Fig. 2B). The activities of Esc(1-18) and H2 against *A. baumannii* were also very rapid (killing times, 2 to 5 min),
while the activities of the temporins against the same species were quite slow (killing times, ≥30 min) (Fig. 2C and D). Finally, *P. aeruginosa* was not killed by any of the peptides used at the bactericidal concentrations after 30 min of incubation (Fig. 2E), while a bactericidal effect was evident after 10 to 30 min of incubation when the peptides were used at twofold the bactericidal concentrations (Fig. 2F).

**DISCUSSION**

The possibility of using naturally occurring AMPs or their derivatives alone or in combination with conventional antibiotics in clinical practice is a measure increasingly being taken into consideration (13, 24). Among the AMPs from the genus *Rana*, *T A* has been the more widely investigated...
for its antibacterial activity both in vitro (11) and in vivo (6) and for its mechanism of action (36). Far less investigated are the spectra of activity of other amphibian peptides, especially against clinically relevant species, under standard conditions and in the presence of biological fluids, such as serum.

In the present study, a comparative analysis of the bactericidal activities of five AMPs from three different amphibian species against multidrug-resistant strains isolated from hospitalized patients was carried out. Notably, in contrast to the majority of natural AMPs, which contain high numbers of positively charged amino acids, temporins and bombinins H represent two families of short and mildly cationic peptides that bear only one single and two basic amino acids, respectively. When they were tested in buffer, all the peptides were found to be endowed with bactericidal activity against a wide range of bacterial species and strains, but with some differences. The temporins were quite active against the majority of the strains of the two gram-positive species tested (E. faecium and S. aureus), while relatively high concentrations were needed to obtain a bactericidal effect against most of the strains of the gram-negative species S. maltophilia and P. aeruginosa. Moreover, when they were studied in time-kill experiments, the temporins tended to act more quickly against gram-positive bacteria than gram-negative bacteria. These results are in agreement with those of our previous studies that have demonstrated that temporins are, in general, more active against gram-positive bacteria than gram-negative bacteria (19, 29). It has recently been emphasized that, unlike temporin L, T$_A$ and T$_B$ oligomerize when they come into contact with the anionic LPS (29). It is likely that such aggregates cannot diffuse efficiently through the bacterial cell wall to reach and permeate the target plasma membrane, and this provides a possible explanation for the relatively low levels of activity of T$_A$ and T$_B$ against the gram-negative species. However, it is interesting to note that the three strains of the gram-negative A. baumannii, as well as one of the S. maltophilia strains (strain 2), were sensitive to temporins at a concentration range similar to or even lower than that required to kill E. faecium and S. aureus. This finding suggests that subtle differences in the compositions of LPS or other surface molecules may influence the susceptibilities of the different gram-negative species/strains to temporins.

The activity of Esc(1-18) against a number of gram-positive and gram-negative reference laboratory strains or isolates obtained from humans and plants has been investigated previously (16). In the present study, the peptide was demonstrated to kill multidrug-resistant gram-positive species at intermediate to low concentrations; but its activity against all three gram-negative species analyzed was particularly strong, and it was active at concentrations far lower than those of the other peptides tested. Moreover, the bactericidal effect of Esc(1-18) against the two gram-positive species was quite slow, while it killed the gram-negative bacteria, especially S. maltophilia and A. baumannii, within a few minutes.

No bias toward any of the bacterial species tested was observed when the antibacterial properties of H2 were analyzed. The peptide also exerted quite fast killing kinetics against both gram-positive and gram-negative species, with the only exception being S. aureus, which was killed, but not earlier than 30 min.

Among the bacterial species tested, the S. maltophilia strains showed the highest strain-to-strain variability in their susceptibilities to the temporins (T$_A$, T$_B$, and T$_C$) and H2, with bactericidal concentrations varying over a range of 4 dilutions. Such interstrain variability did not seem to be related to the phenotypes of resistance to conventional antibiotics exhibited by the three strains, as they were very similar for all of them. Rather, it may have been due to intrinsic differences in the compositions of the surface molecules among isolates of this species, which may interfere with the interactions between temporins and H2 and the bacterial surface and, in turn, determine the various degrees of susceptibility to these peptides.

Interestingly, this paper shows that the target selectivities of the AMPs derived from three different species of frogs against multidrug-resistant clinical isolates of gram-negative and gram-positive bacteria were different. Our findings also correlate with previous results obtained with microorganisms belonging to the natural flora of these frogs, which includes mainly gram-negative species, such as Acinetobacter junii, Aeromonas hydrophila, Enterobacter agglomerans, and Klebsiella pneumoniae (4). Indeed, when the peptides were tested on single bacterial species of the frog microbial flora, Esc(1-18) was generally the most potent peptide, whereas the temporin analogues displayed only weak antimicrobial activities (16, 21, 23).

One of the major limits of the use of AMPs as new therapeutic drugs is their possible inhibition by serum and/or other biological fluids (24, 37). For this reason, to date, the most feasible form of application of AMPs seems to be as topical or aerosol agents rather than systemic agents (40). Very few studies have investigated the in vitro antimicrobial activities of amphibian peptides or their derivatives under conditions more closely related to the in vivo situation, e.g., in the presence of complex biological fluids such as human serum (19). Interestingly, we have previously demonstrated that T$_A$ and T$_B$ maintain their activities against some gram-positive bacterial species in 33% heat-inactivated human serum (19). In agreement with the findings of studies performed with peptides of human or other origin (15, 24, 37), the results obtained in this study demonstrated that 20% serum was able to inhibit to a certain extent the antibacterial properties of the amphibian peptides under investigation. Nevertheless, by using concentrations from 2 to 16 times higher than those able to give a bactericidal effect in buffer, all AMPs showed bactericidal effects against gram-positive species in the presence of serum. In contrast, the activities of all the peptides except Esc(1-18) against gram-negative species were strongly inhibited by 20% serum. We have also previously noted a stronger inhibitory effect of human serum on the activity of an AMP, the human peptide beta-defensin-3, against gram-negative bacteria than against gram-positive bacteria (15). It may be argued that serum components (i.e., LPS-binding proteins) may preferentially bind to the surfaces of gram-negative bacteria, hampering the interaction of the peptides with their main target, the cytoplasmic membrane. One of the most striking findings of this study was the very strong and fast bactericidal activity of Esc(1-18) against gram-negative species, which was also maintained at relatively low concentrations in the presence of human serum.
A major obstacle to the development of therapeutically useful antimicrobial agents based upon amphibian skin secretions is their toxicity, particularly if they have to be administered systemically (7). Previous studies have demonstrated that when Esc(1-18) was tested a concentrations up to 16 μM, it had a reduced cytolytic activity against human erythrocytes compared with that of the full-length peptide (16). Therefore, the potent and fast antibacterial activity of Esc(1-18) (particularly against gram-negative bacteria), the preservation of this activity in serum, and the absence of hemolytic activity at bactericidal concentrations make this peptide a promising candidate for use in the design of new antimicrobial agents. Studies aimed at testing the antibacterial activity of Esc(1-18) in animal models of sepsis or in association with conventional antibiotics against nosocomially relevant species will contribute to a better evaluation of its real therapeutic value for clinical application.

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