In Vitro Activity and Killing Effect of Uperin 3.6 against Gram-Positive Cocci Isolated from Immunocompromised Patients

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The in vitro activity of uperin 3.6, alone or combined with six antibiotics, against gram-positive cocci, including Rhodococcus equi, methicillin-resistant staphylococci, and vancomycin-resistant enterococci, was investigated. All isolates were inhibited at concentrations of 1 to 16 mg/liter. Synergy was demonstrated when uperin 3.6 was combined with clarithromycin and doxycycline.

Overuse of antibiotics and failure to apply basic infection control policies and procedures have contributed to the increasing multidrug resistance of many nosocomial pathogens (4, 9, 14). It represents a serious clinical problem and has created a need for the development of new antimicrobial agents to treat these infections. With the appearance of methicillin-resistant (MR) and vancomycin-resistant (VR) gram-positive pathogens, the need for different therapeutic agents is greatly increased, and it has become critical to identify effective agents to treat multidrug-resistant gram-positive infections with novel mechanisms of activity (4, 12, 13, 14, 16, 20).

During the past decade, a large number of antimicrobial peptides from different organisms have been isolated and characterized (1, 2, 10, 11, 17, 19). One major area of targeted research has been the skin secretions from amphibians, which have yielded a rich variety of bioactive molecules. The structural diversity of polypeptides secreted from amphibian dermal granular glands is probably reflective of a plethora of different biological functions, including the regulation of skin physiology, defense against predators, or prevention of skin colonization/infection by microorganisms (1). Of particular note has been the focused effort, during the last decade, of several studies which have reported the host defense peptides exuded in secretions from dorsal glands of some 20 species of Australian anurans, including species from the genera Litoria and Uperoleia (3, 5, 18). Uperin 3.6 is a wide-spectrum antibiotic peptide isolated from the Australian toadlet Uperoleia mjobergi. With only 17 amino acid residues, uperin 3.6 is smaller than most other wide-spectrum antibiotic peptides isolated from amphibians. In 50% trifluoroethanol, it adopts a well-defined amphipathic alpha helix with distinct hydrophilic and hydrophobic faces. Examination of the activities of synthetic modi-

![FIG. 1. Time-kill kinetics of uperin 3.6 against the following quality control strains: MS S. aureus ATCC 29213, MR S. aureus ATCC 43300, VS E. faecalis ATCC 29212, VR E. faecalis ATCC 51299, R. equi ATCC 6939, and S. pyogenes ATCC 19615.](http://aac.asm.org/...)}
enterococci, as well as to investigate its in vitro interaction with five clinically used antibiotics.

**Organisms.** The quality control strains included methicillin-susceptible (MS) *Staphylococcus aureus* ATCC 29213, MR *S. aureus* ATCC 43300, vancomycin-susceptible (VS) *Enterococcus faecalis* ATCC 29212, VR *E. faecalis* ATCC 51299, *R. equi* ATCC 6939, and *Streptococcus pyogenes* ATCC 19615. Twenty nosocomial isolates of each species were tested with the exception of VR *E. faecalis* (12 isolates) and *R. equi* (12 isolates). The isolates were obtained from distinct immunocompromised patients coming from central Italy and admitted to the Hospital Umberto I, Ancona, Italy. Identification of the strains was performed according to standard procedures. The identification was confirmed by means of the API-Strep and API-Staph systems (bioMérieux Italia, Italy). The different API codes and susceptibility patterns stated the independence of all isolates.

**Antimicrobial agents.** Uperin 3.6 (Gly-Val-Ile-Asp-Ala-Ala-Lys-Lys-Val-Val-Asn-Val-Leu-Lys-Phe-NH₂) was synthesized by Fmoc (9-fluorenylmethoxycarbonyl) solid-phase chemistry by the Faculty of Pharmacy, Medical University of Gdańsk, Gdańsk, Poland (7). The peptide was purified by high-pressure liquid chromatography on a Knauer K501 two-pump system and analyzed by chemical analysis, namely, matrix-assisted laser desorption ionization–time of flight mass spectrometry.

Vancomycin and doxycycline (Sigma-Aldrich, Milan, Italy), imipenem (Merck, Sharp & Dohme, Milan, Italy), clarithromycin (Abbott, Rome, Italy), quinupristin-dalfopristin (Aventis Pharma, S.p.A., Lainate, Milan, Italy), and linezolid (Pharmacia S.p.A., Milan, Italy) were tested as control agents.

**MIC and MBC determinations.** MICs and minimum bactericidal concentrations (MBCs) were assayed according to the procedures outlined by the Clinical and Laboratory Standards Institute (formerly NCCLS) (15). The ATCC strains mentioned above were used as controls. Experiments were performed in triplicate.

**Bacterial killing assay.** The ATCC control strains were used to study the in vitro killing effect of uperin 3.6. Aliquots of exponentially growing bacteria were resuspended in fresh Mueller-Hinton broth at approximately 10⁷ cells/ml and exposed to uperin 3.6 at 2× MIC for 0, 5, 10, 15, 20, 25, 30, 40, and 50 min at 37°C. After these times, samples were serially diluted in 10 mM of sodium HEPES buffer (pH 7.2) to minimize the carryover effect and plated onto Mueller-Hinton agar plates to obtain viable colonies (8).

**Synergy studies.** Six strains of MR *S. aureus*, six of VR *E. faecalis*, six of *R. equi*, and six of *S. pyogenes* were used to test the antibiotic combinations by a checkerboard titration method. The fractional inhibitory concentration index was calculated according to the following equation: FIC index = FICₐ + FICₐ = A/MICₐ + B/MICₐ, where, respectively, *A* and *B* are the MICs of drug *A* and drug *B* in the combination, MICₐ and MICₐ are the MICs of drug *A* and drug *B* alone, and FICₐ and FICₐ are the FICs of drug *A* and drug *B*. The FIC indexes were interpreted as follows: <0.5, synergy; 0.5 to 4.0, indifferent; and >4.0, antagonism (6).

All isolates were inhibited by uperin 3.6 at concentrations of 1 to 16 mg/liter. For the control strains *S. aureus* ATCC 29213, *S. aureus* ATCC 43300, *E. faecalis* ATCC 29212, *E. faecalis* ATCC 51299, *R. equi* ATCC 6939, and *S. pyogenes* ATCC

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The aim of the present study was to evaluate the in vitro activity of uperin 3.6 and its bactericidal effect for a large number of gram-positive cocci, including *Rhodococcus equi*, methicillin-resistant staphylococci, and vancomycin-resistant...
19615, the peptide exhibited MICs of 8, 8, 16, 16, 8, and 4 mg/liter and MBCs of 16, 16, 32, 32, 16, and 8 mg/liter, respectively. The results are summarized in Table 1.

Killing by uperin 3.6 was rapid, and its activity on staphylococci was complete after a 30-min exposure period at a concentration of 2× MIC, its activities on R. equi and enterococci were complete after 40 min, and its activity on S. pyogenes ATCC 19615 was complete after 10 min at the same concentration (Fig. 1).

In the combination studies, synergy was observed only for a combination of uperin 3.6 and doxycycline or clarithromycin. (Table 2).

Overall, our data showed that most of the antimicrobials tested were more active than uperin 3.6 for each genus. Nevertheless, uperin 3.6 was equally active against both susceptible and multiresistant clinical isolates. Of interest, our data showed that although uperin 3.6 was less active than control agents against R. equi isolates, it exhibited MBCs greatly lower than those of other agents. This finding could be very interesting in consideration of the fact that, despite the good in vitro activities of traditional antibiotics, therapy is often partially effective, and relapses occur during the course of the disease. Moreover, time-kill studies have shown a rapid bactericidal effect even if the inactivation of E. faecalis and R. equi appears to be slower than that observed for the other gram-positive cocci.

Combination studies showed that uperin 3.6 acted synergistically with hydrophobic antibiotics, although these data were obtained by using a limited number of isolates. The antimicrobial activities of macrolides and tetracyclines result from their interactions in initiating membrane association of cationic peptides, as well as their selective toxicities toward microorganisms, opinions with regard to the subsequent events that eventually lead to the lysis of microbes differ. Most cationic peptides kill microorganisms by directly permeabilizing and lysing cell membranes, although some are thought to inhibit synthesis of DNA, RNA, and cellular proteins and to insert themselves into cytoplasmic membrane, triggering the activity of bacterial murein hydrolases and leading to degradation of the peptidoglycan with lysis of the cell.

The good antimicrobial activities, as well as the synergistic interactions with hydrophobic antibiotics, suggest uperin 3.6 as a promising candidate for potential application in the treatment of gram-positive bacterial infections. Further in vivo studies are required to validate these results.

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REFERENCES

9. Gonzalez, A., T. Bischof, S. Tallent, G. Sheke, B. Ostrowski, M. B. Edmond,