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Wide-Spectrum Antibiotic Activity of Synthetic, Amphipathic Peptides

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PGYa and PGAa are synthetic, amphipathic, α-helical peptides that were designed using a novel "sequence template" approach. Their antimicrobial activity was tested against several pathogenic clinical isolates, most of which were multiply resistant to conventional antibiotics. PGYa appeared to be more active towards Gram-positive species (MIC = 0.5-4 μM), towards such Gram negative species as P. aeruginosa, X. maltophilia, E. coli, K. pneumoniae and S. enteritidis (MIC = 1-4 μM), and towards the filamentous fungus A. niger (MIC = 8 μM). Conversely, PGAa showed the greater activity towards three Candida species (MIC = 2-16 μM). The peptides were shown to have a bactericidal activity, resulting in a decrease of viability for both Gram-positive and -negative bacteria of 3-6 logs within 60 min. Scanning electron microscopy of S. aureus and E. coli treated with PGYa shows considerable roughening and blebbing of the bacterial surfaces providing conclusive evidence that the peptide is membrane active.

Investigations on host-defence biology, or "innate immunity", have disclosed an important class of antibacterial agents consisting of endogenously produced, gene-encoded cationic peptides. Several different types of linear peptides, and of peptides cyclized via one or more disulphide bridges, have been reported (1-7). These molecules generally have quite a broad range of antibacterial and, in some cases, antifungal activity and act rather rapidly. Like polymyxins, they apparently do not interact with specific metabolic targets but rather bind to cell membranes and perturb their permeability (8-13).

In studies aimed at investigating the mechanism of action of the natural products and/or at improving on their antimicrobial activity, numerous artificial peptides have been synthesized, following various approaches (9, 12, 14-18). These are based principally on synthesis of substitution analogs or on analogues with sequences dictated by physicochemical characteristics. Some of the synthetic analogues have also been evaluated as potential novel antibiotics, particularly for use in topical and parenteral formulations (19).

We have designed and synthesized linear amphipathic antimicrobial peptides using a novel "sequence template" approach, which consists in extracting sequence patterns after comparison of a large series of natural counterparts (12), selected from sequences present in a purposely constructed database[2]. Of the newly designed peptides, the 22-residue PGYa has a net charge of 7+ and a sequence similarity with a group of antimicrobial peptides belonging to the mammalian cathelicidin family, while the 20-residue PGAa has a net charge of 8+ and includes sequence features of several insect and frog antimicrobial peptides. Both peptides have a high potential for assuming an α-helical conformation in anisotropic media.

In this paper we describe the antibacterial and antifungal activity of PGYa and PGAa against a wide variety of Gram-positive and Gram-negative bacteria and fungi collected from immune suppressed patients, following disease or chemotherapy, or from patients suffering from cystic fibrosis or other chronic diseases. The microorganisms here investigated can cause infections at sites where treatment with this type of peptide antibiotic could be feasible, and this study is thus an initial evaluation of the peptides as lead compounds for potential therapeutic agents.

MATERIALS AND METHODS

Peptides. PGYa (GLLRRLRDFLKKIGEKFKKIGY-NH_2, MW = 2678 Da) and PGAa (GILSKLGKALKKAAKHAAKA-NH_2, MW = 2003 Da) were prepared by solid-phase synthesis, purified and characterized by analytical RP-HPLC and electrospray mass spectrometry, as described by Tossi et al. (7, 12). Helical net and wheel repre-

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sentations of the peptides are shown in Fig. 1, and were prepared using the Peptide Companion program (Coshi-Soft).

Microorganisms and growth conditions. Bacterial clinical isolates were provided by the Institute of Hygiene of the University of Trieste. Resistance to antibiotics was determined using a Becton Dickson AutoSceptor system supplemented by agar diffusion assays that utilized panels of antibiotics suggested by NCCLS standards (20) and the clinical situation. Bacteria were grown in Mueller-Hinton broth (Difco Labs, Detroit, MI) at 37°C to the stationary phase. Subcultures of exponentially growing bacteria, quantified by visual comparison to 0.5 McFarland barium sulphate standard (Bio Merieux, France) and verified by subsequent plating and colony counting, were used for the antimicrobial assays.

Fungi were also isolated in the Institute of Hygiene, and cultivated as recommended by the NCCLS method M27-T (21). Yeast isolates were cultivated in solid Sabouraud agar dextrose medium (Oxoid, England) for 24-48 hours in Petri dishes, while *A. niger* was cultivated in the same medium for 7 days in slants. Yeasts were quantified by determining the turbidity at 530 nm, adjusted by comparison to 0.5 McFarland barium sulphate standard, while *A. niger* samples were prepared by adjusting conidial suspensions to about 80% transmission at 530 nm.

All microorganisms were stored in 15% glycerol at -20°C to permit repeated susceptibility tests.

Antimicrobial assays. MICs were determined in Mueller-Hinton broth by the microdilution susceptibility test (7). PGYa or PGAa were serially 1:1 diluted, in duplicate, in the wells of 96-well round bottom microtiter plates, starting from a concentration of 32 μM (83 μg/ml and 64 μg/ml respectively). Aliquots (50 μl) of bacterial suspensions (1-5 $\times$ 10^5 CFU/ml) were added to each well and the plates were incubated overnight at 37°C. MIC values were the mean of at least three independent experiments. Organisms were considered to be susceptible if growth was prevented at a peptide concentration of 32 μM or less. MIC values with fungi were determined in RPMI-1640 medium (Biochrom, Berlin) buffered to pH 7.0 with 0.165 M morpholinepropanesulphonic acid, following the M-27 method of the NCCLS (21), scaled to perform microdilution assays.

The bactericidal activity of the two synthetic peptides was tested against selected isolates by incubating exponentially growing bacteria (10^6 – 10^7 CFU/ml) in phosphate-buffered saline, pH 7.4, at 37°C, with peptide at 16 μM. At different times, 50 μl of the bacteria/peptide suspensions were serially diluted several fold in ice-cold phosphate-buffered saline, and each dilution plated on nutrient agar in duplicate and incubated overnight to allow colony counts by direct inspection of the plates.

Scanning electron microscopy (SEM). Exponentially growing *E. coli* or *S. aureus* were incubated in phosphate-buffered saline (10^7 CFU/ml) at 37°C in the presence of 10 μM peptide. At different times (2-60 min) 150 μl aliquots of the cells were fixed with an equal volume of 5% glutaraldehyde in 0.2 M sodium cacodylate buffer, pH 7.4. Controls were run at t = 0 in the absence of the peptide. After fixation for 2 hrs, the bacteria were collected on a Nucleopore filter (pore size 0.2 mm), and washed at least three times with 0.1 M cacodylate buffer. They were then treated on the filters with 1% osmium tetroxide, washed with 5% sucrose in 0.1 M cacodylate buffer, and subsequently dehydrated with a graded ethanol series. The samples were vacuum dried and mounted onto aluminum SEM mounts. After sputter coating with gold, they were analyzed on a Leica Steroscan 430i instrument (Leica Inc. Deerfield, IL).

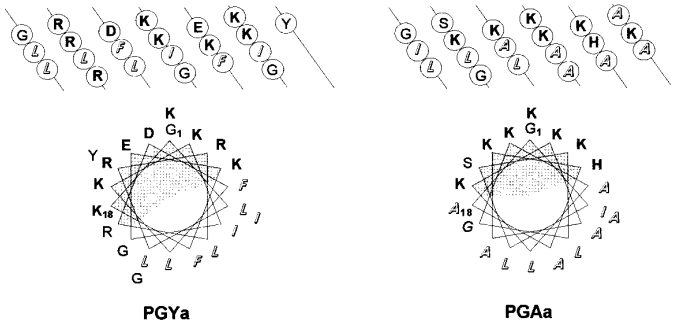

FIG. 1. Helical net and wheel representations of PGYa and PGAa. Charged residues are bold and hydrophobic residues are outlined; the polar sector is shadowed.

TABLE 1

MICs of PGYa and PGAa against Gram-Positive Bacteria

Organism[a]	Resistances[b]	MIC (μM) PGYa	PGAa
S. epidermidis	1–4, 6, 7, 9, 10, 14, 15, 17–19, 22	1	8
S. aureus	1, 4, 7, 9, 12, 13, 16	1	32
	1–7, 9, 10, 14, 15, 17–21	1	16
	n.t.	1	32
	n.t.	2	16
S. beta-hemolytic gr. A	n.t.	1	4
S. agalactiae B	19	1	4
E. faecalis	11–13, 16, 19	4	16
E. faecium	8, 15, 18	0.5	4
	n.t.	1	16
Bacillus sp.	n.t.	2	4

[a] Each row concerns a different clinical isolate; [b]Only determined resistances are shown: 1) penicillin; 2) oxacillin; 3) cefazolin; 4) erythromycin; 5) rifampicin; 6) clindamycin; 7) amoxicillin/clavulanic; 8) ampicillin; 9) ampicillin/sulbactam; 10) cephalothin; 11) cefotaxime; 12) cefamandole; 13) ceftazidime; 14) ceftriaxone; 15) imipenem; 16) amikacine; 17) gentamycin; 18) netilmicin; 19) tetracycline; 20) ciprofloxacin; 21) ofloxacin; 22) trimethoprin/sulfa 1/19; n.t.: not tested.

RESULTS AND DISCUSSION

PGYa and PGAa are synthetic peptides designed using a novel "sequence template" approach (12) so as to pack highly cationic, amphipathic and helical structures into relatively short sequences (20-22 residues) (Fig. 1). Their antibacterial activity against a variety of bacterial clinical isolates, in most cases resistant to several conventional antibiotics, was evaluated by determining the MIC values. All the Gram-positive bacterial species tested (Table 1) were found to be inactivated by both PGYa and PGAa, although generally with different susceptibilities. PGYa showed consistently lower MIC values (0.5-4 μM) and was particularly potent against *Staphylococcus* strains (MIC $\leqslant$ 2 μM), including those multiply resistant to antibiotics. PGAa was found to be generally less active, and also showed a less homogeneous behaviour. The difference in activity to PGYa is particularly marked with *S. aureus* isolates.

Gram-negative bacteria in general showed a comparable behaviour with both peptides, and not all species

TABLE 2

MICs of PGYa and PGAa against Gram-Negative Bacteria

Organism[a]	Resistances[b]	MIC (μM) PGYa	MIC (μM) PGAa
B. cepacia	7–14, 16, 17, 19–24, 27–29, 31, 33, 34	16	>32
	7–10, 12, 14–19, 27–29, 31, 32	16	>32
	7–10, 12, 14, 16–19, 28, 31, 32	16	>32
	7–12, 14, 15–17, 19–24, 27–29, 31–33	32	>32
P. aeruginosa	7–10, 12, 16, 17, 19–22, 27, 28, 31–33	4	4
	7–19, 21–33	4	4
(m)[c]	7–12, 14, 15, 19–22, 27, 28, 32, 33	2	4
(m)	7–12, 14, 19–22, 27, 28, 32, 33	2	4
(m)	7–10, 12, 14, 19, 21, 22, 27, 28, 32, 33	1	2
X. maltophila (m)	7–19, 21, 23, 24, 26–33	1	8
	7–17, 19–21, 23–25, 28, 29, 31–33, 35	4	4
E. coli	7–10, 13, 14, 22–24, 29, 31, 36	2	8
K. pneumoniae	8–10, 23, 24, 34	1	8
Enterobacter sp.	7, 10, 28	8	8
S. enteritidis	n.t.	1	1
S. marcescens	7–10, 12, 13, 19, 28, 32	>32	>32
P. mirabilis	19, 22, 32	>32	>32
M. morganii	7–10, 23, 28, 34, 35	>32	>32
P. stuartii	7–10, 17, 22, 31, 34, 35	>32	>32

[a] Each row concerns a different clinical isolate; [b]Only determined resistances are shown: 7) amoxicillin/clavulanic; 8) ampicillin; 9) ampicillin/sulbactam; 10) cephalothin; 11) cefotaxime; 12) cefamandole; 13) ceftazidime; 14) ceftriaxone; 15) imipenem; 16) amikacine; 17) gentamycin; 18) netilmicin; 19) tetracycline; 20) ciprofloxacin; 21) ofloxacin; 22) trimethoprin/sulfa 1/19; 23) mezlocillin; 24) piperacillin; 25) piperacillin/tazobactam; 26) ticarcillin/clavulanic; 27) cefotetan; 28) cefoxitin; 29) aztreonam; 30) meropenem; 31) tobramycin; 32) chloramphenicol; 33) pefloxacin; 34) nitrofurantoin; 35) trimethoprin; 36) ceftizoxime; n.t.: not tested. [c](m) indicates a mucoid isolate.

were susceptible (Table 2). The *B. cepacia* isolates, for example, showed MIC values > 32 μM with respect to PGAa, and of 16-32 μM with respect to PGYa. On the other hand, several *Pseudomonaceae* nonmucoid and mucoid strains (*P. aeruginosa* and *X. maltophilia*) were found to be susceptible to both peptides (MIC 1-8 μM). These results are significant, as *P. aeruginosa* is well known for its intrinsic resistance to a large variety of antimicrobials (see Table 2) due to a low permeability of the outer membrane as well as an active efflux system (22), and is considered a potential source of hospital infections. Of the *Enterobacteriaceae, E. coli, K. pneumoniae, Enterobacter sp.* and *S. enteritidis* were also found to be quite susceptible to both peptides (MIC 1-8 μM), while *S. marcescens* and the *Proteae, P. mirabilis, M. morganii and P. stuartii,* were not susceptible to either. In the case of *P. mirabilis,* resistance to cationic antimicrobial peptides has been ascribed to an outer surface which has a less anionic lipopolysaccharide than other Gram-negative species, and thus has a weaker initial electrostatic interaction with the peptides (12). A relative resistance towards cationic antimicrobial peptides reported for *Proteus vulgaris* (9), has similarly been ascribed to a lower content of anionic phospholipids in the cytoplasmic membrane.

The two peptides were found to be rapidly bactericidal for exponentially growing, multi-drug resistant Gram-negative bacteria such as *X. maltophilia,* and *P.*

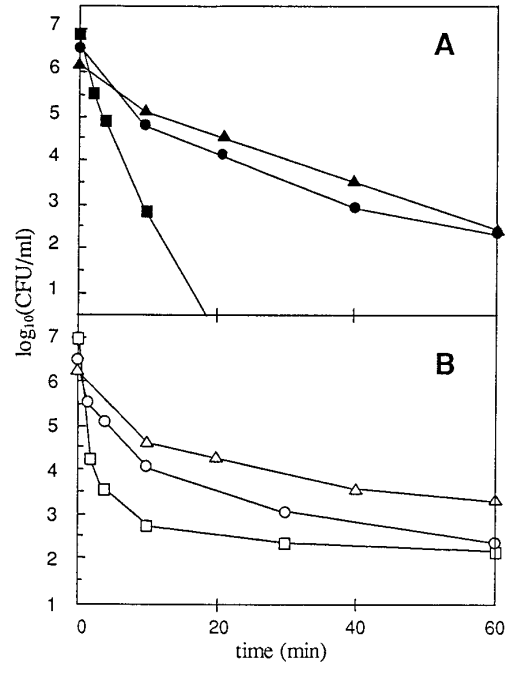

FIG. 2. Time killing plots for PGYa (A) with *S. aureus* (▲), *S. epidermidis* (●), *X. maltophilia* (■) and for PGAa (B) with *E. faecalis* (△), *K. pneumoniae* (○) and *P. aeruginosa* (□), at 16 μM peptide. CFU = colony forming units.

aeruginosa, whereas susceptible Gram-positive bacteria such as *S. aureus* and *E. faecalis* are killed more slowly (Fig. 2). The decrease in viable bacteria was in any case 3-6 logs values within the time limit of the assays. The difference in killing kinetics for Gram-negative and Gram-positive bacteria may be ascribed to a possible interference of teichoic and teichuronic acids, and other anionic groups, on the thick external peptidoglycan layer of the latter category, which must be traversed by the cationic peptides before reaching the cytoplasmic membrane.

SEM of *E. coli* or *S. aureus* treated with PGYa show a considerable roughening of the bacterial membranes and formation of blebs on the cell surface (Figs. 3B and 3D), in contrast to the smooth surfaces of untreated bacteria (Figs. 3A and 3C). This is a strong indication that the membrane is being considerably modified by the peptide, which may be correlated to bacterial inactivation, and provides morphological evidence that this type of amphipathic, α-helical peptides significantly alter bacterial membranes (12, 18).

The antimicotic activity of the synthetic peptides was also assayed against several yeasts and one filamentous fungus, as shown in Table 3. Both peptides were quite active against *C. neoformans;* PGAa in particular showed a good activity towards three *Candida* species. Only *C. glabrata* was found not to be susceptible to either peptide (MIC $>$ 32 μM). In contrast, the filamentous fungus *A. niger* was found to be susceptible only to PGYa.

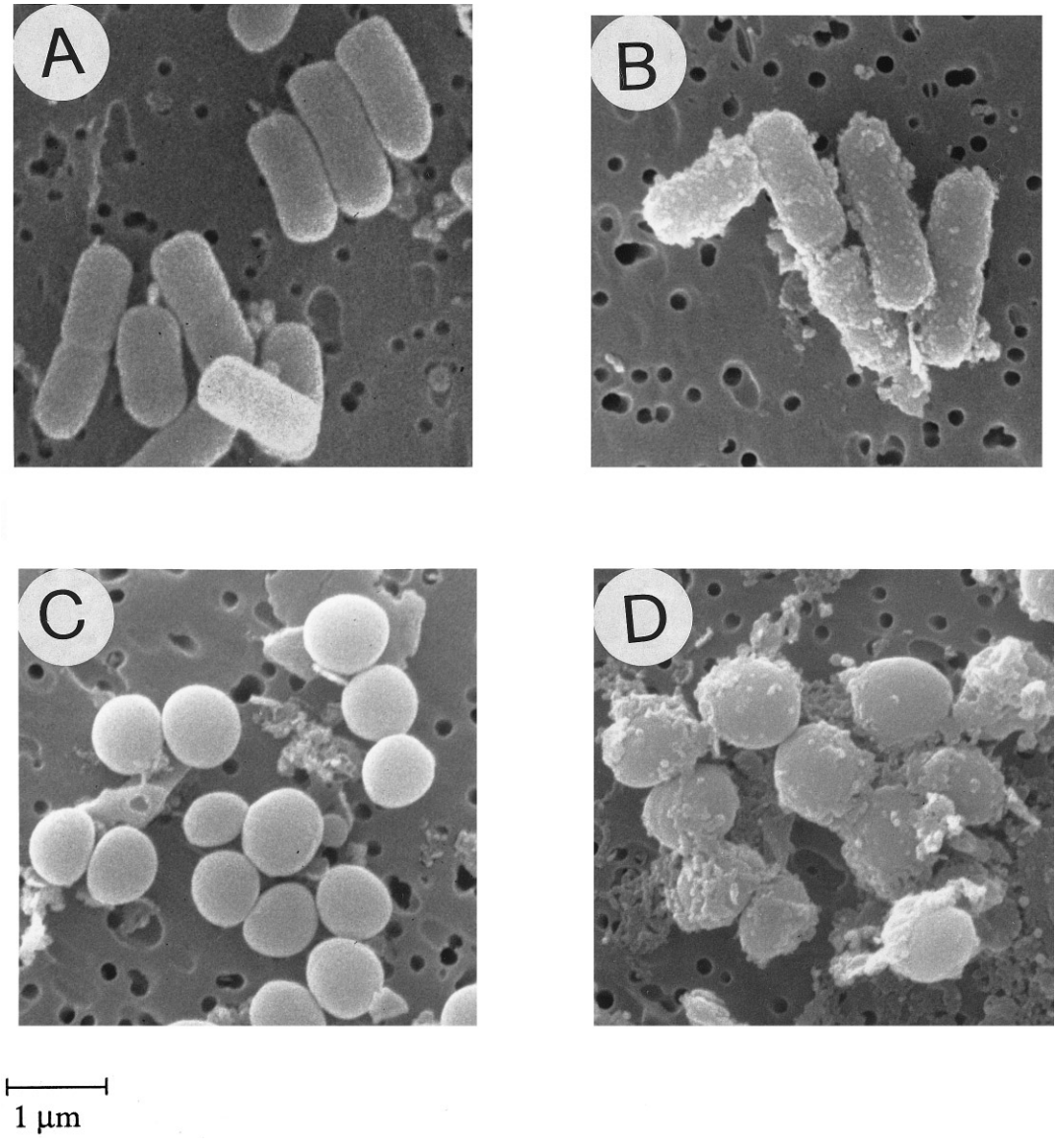

FIG. 3. Scanning electron micrographs of *E. coli* (top) and *S. aureus* (bottom) before (A and C) and after (B and D) treatment for 30 min with 10 μM PGYa.

TABLE 3

MICs of PGYa and PGAa against Some Fungi

Organism	MIC (μM)	
	PGYa	PGAa
C. albicans	8	4
C. parapsilosis	8	2
C. tropicalis	16	8
C. glabrata	>32	>32
C. neoformans	2	2
A. niger	8	>32

PGYa and PGAa have a similar size and charge so that any difference in interaction with bacteria and fungi must derive from other biophysical characteristics. The generally greater activity of PGYa than PGAa towards both Gram-positive and -negative bacteria may derive from a favourable contribution of the particularly high amphipathicity of the former peptide and/or from an unfavourable contribution deriving from the broader hydrophobic sector of PGAa (Fig. 1). PGAa is somewhat more toxic than PGYa on eukaryotic (animal) cells, while less active against a limited panel of standard bacterial strains (12). This is in line with the present results, which show that PGAa is more potent against yeast cells while generally less potent against bacteria than PGYa. Again, an explanation may come from the different degrees of amphipathicity and helicity of the two peptides, and the make-up of the hydrophobic face on the helix. Thus, the higher hydrophobic moment (amphipathicity) of PGYa may favour interaction with prokaryotes, while the wider hydrophobic sector and more marked helicity of PGAa (due to the high content of alanines), may favour its interaction with eukaryotic cell membranes (15, 23). *A. niger* appears to be the only firm exception, possibly due to its particular cell wall make-up.

In conclusion, we have demonstrated that two designed, amphipathic antimicrobial peptides, PGYa and PGAa, are quite potent at inactivating several pathogenic bacteria, both Gram-positive and Gram negative, and fungi. Most of the microorganisms which are susceptible to these peptides are not only resistant to a variety of common antibiotics but are also important opportunistic or pathogenic organisms (e.g., *Pseudomonas* and *Aspergillus* in cystic fibrosis, *Aspergillus* in keratitis and *Candida* and *Cryptococcus* in immune suppressed individuals). Therefore, our synthetic peptides might be considered potential lead compounds for the development of peptide antibiotics for use in the treatment of selected pathologies.

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