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In vitro activity and mode of action of diastereomeric antimicrobial peptides against bacterial clinical isolates

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***Objectives*: Increasing resistance of pathogenic bacteria to antibiotics is a severe problem in health care and has intensified the search for novel drugs. Cationic antibacterial peptides are the most abundant antibiotics in nature and have been frequently proposed as new anti-infective agents. Here, a group of diastereomeric (containing D- and L-amino acids) peptides is studied regarding their potency against multiply resistant clinical isolates and their modes of action against Gram-positive cocci.**

***Methods*: MIC determinations and chequerboard titrations followed established procedures. Mode of action studies included killing kinetics and a series of experiments designed to characterize the impact of the diastereomeric peptides on bacterial membranes.**

***Results*: The tested diastereomers displayed high antimicrobial and broad spectrum activity with amphipathic-2D being the most active peptide. Synergic activities were observed with individual strains. Mode of action studies clearly demonstrated that the cytoplasmic membrane is a primary target for the peptides and that membrane disruption constitutes a significant bactericidal activity for the major fraction of a bacterial population. However, depending on the indicator strain, the results also suggest that additional molecular events contribute to the overall activity.**

Keywords: synergic activities, membrane permeabilization, intracellular targets

Introduction

In the past decade, many cationic antimicrobial peptides (AMPs) of various structural classes have been isolated from a wide range of animal, plant and bacterial species.[1–4] The relevance of cationic AMPs as important components of innate immunity in all species has become increasingly apparent. The peptides not only provide a rapid first line of antibiotic defence against invading pathogens, but many mammalian peptides also have receptor-mediated effector functions in chemotaxis. Antimicrobial defence peptides are ancient elements and their induction pathways in all organisms are conserved.[5] The gene-encoded polypeptides are mostly composed of <60 amino acid residues and carry a positive net charge.[6] Despite significant variations in composition, length and secondary structure,[7] most AMPs adopt an amphipathic conformation in a hydrophobic environment, which in addition to the positive charge is believed to play a key role in the mode of action.[8] The hydrophilic, cationic part is proposed to initiate electrostatic interaction with the negatively charged components of the membrane of microbes; the hydrophobic portion is supposed to permit the peptides to insert into and permeate the membrane.[9,10] The positive charge provides some degree of selectivity towards negatively charged microbial cell envelopes and cytoplasmic membranes.

The increasing resistance of bacteria to conventional antibiotics encouraged strong efforts to develop antimicrobial agents with new mechanisms of action. Recently, a variety of *de novo* designed AMPs have been synthesized combining optimized characteristics such as high positive charge, amphipathicity and α-helicity. Unfortunately, high antimicrobial activity was frequently accompanied by relatively high haemolytic activity.[8] Furthermore, all L-amino acid amphipathic peptides are sensitive to degradation and clearance by serum components. In recent studies, D-amino acids were incorporated into cytolytic peptides and although the resulting diastereomers simultaneously lost their α-helical structure and haemolytic activity, they retained high antibacterial activity, thus providing a basis for designing novel peptide antibiotics composed of D- and L-amino acids that are selective to microorganisms.[11,12] The diastereomers were composed of leucine and lysine (15 amino acids), one-third of which were D-amino acids.[13] These peptides had been evaluated with regard to their activity against standard laboratory strains, their haemolytic

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activity, their structural features and mode of action on model membranes, demonstrating their high potential for the development of new target antibiotics.

In this paper, we describe the *in vitro* activities of these diastereomers, on their own and in combination with conventional antibiotics, against a set of clinical isolates. To study the hypothesis that permeabilization of the cytoplasmic membrane is responsible for killing, we correlated cell viability and various cytoplasmic membrane functions under identical experimental conditions. Our data strongly suggest that membrane perturbation represents a major event for killing; however, additional lethal or sublethal events clearly contribute to killing of individual strains.

Materials and methods

Bacterial strains

Eighteen clinical isolates from distinct patients as well as two standard laboratory strains were included in this study: *Staphylococcus simulans* 22; *Micrococcus luteus*; *Enterococcus faecium* (two strains, I-11305b and I-11054); *Staphylococcus haemolyticus*, coagulase-negative (CoNS; I-10925); *Staphylococcus epidermidis*, methicillin-resistant (MRSE; LT1324); *Staphylococcus aureus*, methicillin-susceptible (two strains, 5185 and I-11574); *Staphylococcus aureus*, methicillin-resistant (MRSA; two strains, LT1338 and LT1334); *Citrobacter freundii* (I-11090); *Klebsiella pneumoniae* (I-10910); *Escherichia coli* (two strains, I-11276b and O-19592); *Stenotrophomonas maltophila* (two strains, O-16451 and I-10717); *Pseudomonas aeruginosa* (two strains, 4991 and I-10968); *Candida albicans* (two strains, I-11301 and I-11134). The strains were maintained on Mueller–Hinton (MH) agar or on blood agar.

Growth media

All strains were subcultured weekly on blood agar (Becton Dickinson, Erembodegem, Belgium) or Mueller–Hinton (MH; Oxoid, Basingstoke, UK) agar. For the efflux experiments, PYG medium containing 0.2% Bacto-Peptone (Difco, MI, USA), 0.8% glucose (Merck, Darmstadt, Germany), 0.4% yeast extract (Oxoid), 2 mM potassium phosphate (Merck) (pH 7) was used.

MIC determinations

The determination of MICs was carried out in microtitre plate assays. Since cationic peptides bind to polystyrene, 96-well polypropylene plates (Life Technologies) were used throughout.[14] Binding became particularly obvious at low MICs, e.g. below 1 µM, and was further reduced by coating with bovine serum albumin (BSA). A series of two-fold dilutions in MH broth was prepared from a stock solution of the respective peptide (16 µM). The indicator strains were grown to an optical density (600 nm) of 1.0 in half-concentrated MH broth and diluted 1:10^5 with the same medium. Then 100 µL of this suspension was mixed with 100 µL of the peptide dilution in the well of a microtitre plate. After incubation for 18 h at 37°C, the MIC was read as the lowest concentration of antimicrobial agent resulting in the complete inhibition of visible growth and results given are mean values of three independent determinations.

Chequerboard titrations

Synergic activities were assayed by chequerboard titrations with half-concentrated MH broth. Fractional inhibitory concentration (FIC) indices were calculated as follows: $[(A)/MIC_A] + [(B)/MIC_B] = FIC_A + FIC_B = FIC$ index, where MIC_A and MIC_B are the MICs of drugs A and B when used alone, and (A) and (B) are the MICs of drugs A and B when used in combination. The interaction was defined as synergic if the FIC index was ≤0.5 and antagonistic if the FIC index was >4.0; no interaction was observed if the FIC index was >0.5–4.0.[15]

Killing kinetics

All strains were grown overnight in half-concentrated MH broth and diluted in fresh medium to an optical density of 0.1. Peptides were added in concentrations corresponding to 1, 2 and 4 times the MIC as determined after 18 h. The viable count was monitored up to 22 h. Aliquots were taken at defined intervals, diluted in 10 mM potassium phosphate buffer, and 100 µL of the dilutions were plated in triplicate on MH agar. The plates were incubated at 37°C and the colony forming units (cfu) were counted after 24 h. Since we expected an inoculum effect on the killing kinetics, we carried out MIC determinations not only with the standard inoculum of 10^5 cfu/mL, but also with 10^8 cfu/mL and did not see significant differences between the MIC values, particularly when BSA coated microtitre plates were used.

Efflux of radioactively labelled glutamate

The influence of peptide amphipathic-2D on the uptake and retention of glutamate was investigated as described previously with some modifications.[16] Briefly, strains were cultured in PYG medium at 37°C to an absorbance of 1.0 at 600 nm. Centrifuged cells were resuspended 1:3 in fresh medium, which was supplemented with 100 µg of chloramphenicol per mL to prevent glutamate incorporation. After 15 min of pre-incubation, radiolabelled L-[^{3}H]glutamate (42 Ci/mMol) was added (final concentration 10 µCi/mL), and the culture was immediately divided into two parts. One aliquot was transferred into a flask that already contained amphipathic-2D (10 times the MIC for the respective strain) to tests its effect on the uptake of glutamate; the other part was run as control. After 30 min of incubation, the control was further subdivided into two aliquots, one of which received amphipathic-2D (10 times the MIC) to follow the effect on pre-accumulated amino acids. Samples of 100 µL were filtered through cellulose acetate filters (pore size, 0.2 µm; Schleicher & Schüll, Dassel, Germany) and washed twice with 5 mL of 200 mM potassium phosphate buffer (pH 7) containing 100 µM unlabelled glutamate. Filters were dried and transferred to counting vials filled with scintillation fluid (Quickszint 100; Zinsser, Frankfurt, Germany). The radioactivity was measured in a beta-counter (1900CA; Packard, Downers Grove, IL, USA).

Estimation of the membrane potential

Cells were grown in PYG at 37°C to an absorbance of 1 at 600 nm, centrifuged and resuspended 1:3 in fresh medium. To monitor the membrane potential, 1 µCi/mL of [^{3}H]tetraphenylphosphonium bromide (TPP^+; 26 Ci/mMol) was added. TPP^+ is a lipophilic cation which diffuses across the bacterial membrane in response to a trans-negative $\Delta\psi$. The culture was treated with amphipathic-2D (10 times the MIC for the respective strain) and samples were filtered and washed as described above. Counts were corrected for unspecific binding of [^{3}H]TPP^+ by subtracting the radioactivity of 10% butanol-treated cell aliquots. For calculation of the membrane potential ($\Delta\psi$), TPP^+ concentrations were applied to the Nernst equation [$\Delta\psi = (2.3 \times R \times T/F) \times \log (TPP^+_{in}/TPP^+_{out})$]. A mean $\Delta\psi$ was calculated from a minimum of two independent determinations.

Fluorometric assay for membrane potential

Cells were grown in half-concentrated MH broth to an OD_{600} of 0.5 and incubated for 5 min with 1 µM of the membrane-potential-sensitive fluorescent probe bis-(1,3-dibutylbarbituric acid) trimethine oxonol [$DiBAC_4(3)$; Molecular Probes, Leiden, The Netherlands]. Amphipathic-2D was added at a concentration corresponding to 10 times the MIC.

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Table 1. Sequences of diastereomers

Peptide	Sequence
Amphipathic-1D	L L **K** L L **K** K **L** **L** K K L **L** K L–NH_2
Amphipathic-2D	L L **K** K L **L** K **L** **L** L K L **L** K K–NH_2
Amphipathic-3D	L K **L** L K **K** L **L** **K** K L L **K** L L–NH_2
Scrambled-4D	K L **K** L L **K** L **L** **K** L L K **L** L K–NH_2
Segregated-5D	K K **K** L L **L** L **L** **L** L L L **K** K K–NH_2
Segregated-6D	L L **L** L L **K** K **K** **K** K K L **L** L L–NH_2

Amino acids in bold face are D-enantiomers. All the peptides are amidated in their C-terminus; K = lysine, L = leucine.

Fluorescence was measured at the excitation and emission wavelengths of 492 and 515 nm, respectively.

Results

Antimicrobial activities of the diastereomers

The primary structures of the diastereomers, all of which were amidated at their C terminus and had a charge of +7, are shown in Table 1. All peptides were derived from an α-helical lytic peptide composed of L-lysine and L-leucine (15 amino acids) either by only introducing five D-amino acids or by additional sequence alteration.[13] The antibacterial activity of the peptides was tested against a set of fresh clinical isolates including both Gram-positive and Gram-negative species as well as two *Candida albicans* strains with varying susceptibility patterns. We selected strains with multiple resistances, including those of current clinical concern such as 4-quinolone-resistant Enterobacteriaceae, methicillin-resistant *S. aureus*, VanA-type vancomycin-resistant enterococci and genera of high natural resistance such as *Stenotrophomonas* and *Pseudomonas*. When possible, we included, in addition to two well-characterized laboratory strains, the respective susceptible counterparts. Peptides were tested with a concentration of up to 8 µg/mL corresponding to 4 µM, which is of relevance for the development of a new antibiotic drug.

Peptides amphipathic-1D, -2D and -3D had been designed with a different distribution of the D-amino acids along the hydrophobic and hydrophilic faces. However, all three peptides displayed a similar high antimicrobial and broad-spectrum activity with amphipathic-2D being the most active peptide (Table 2). Interestingly, many of the MICs determined for the clinical strains were significantly lower compared to the MICs which had been obtained for selected type culture collection strains.[13] The activity of peptide scrambled-4D, which has a scrambled sequence of hydrophobic and hydrophilic amino acids, was not significantly changed compared to the amphipathic peptides and equalled amphipathic-1D. Even clustering of the hydrophobic and hydrophilic amino acids as in segregated-5D and -6D preserved activity against several Gram-positive bacteria. The

Table 2. Minimal inhibitory concentrations of the peptides [µM]

Indicator strain	Amphipathic-1D	Amphipathic-2D	Amphipathic-3D	Scrambled-4D	Segregated-5D	Segregated-6D
Staphylococcus simulans	1	1	1	1	1	1
Micrococcus luteus	0.5	0.5	0.5	0.4	0.25	0.25
Enterococcus faecium, I-11305b	4	2	1	>4	>4	>4
Enterococcus faecium, I-11054	>4	>4	>4	>4	>4	>4
CoNS, I-10925	1	1	1	1	1	2
MRSE, LT1324	1	0.5	1	1	1	1
Staphylococcus aureus, 5185	4	2	3	4	>4	4
Staphylococcus aureus, I-11574	4	2	3	4	>4	>4
MRSA, LT1338	>4	4	4	>4	>4	>4
MRSA, LT1334	>4	2	2	4	>4	>4
Citrobacter freundii, I-11090	2	2	2	2	>4	2
Klebsiella pneumoniae, I-10910	4	4	>4	>4	>4	>4
Escherichia coli, I-11276b	2	1	1	1	4	4
Escherichia coli, O-19592	1	1.5	1	1	4	4
Stenotrophomonas maltophila, O-16451	>4	4	>4	>4	>4	>4
Stenotrophomonas maltophila, I-10717	4	2	>4	>4	>4	>4
Pseudomonas aeruginosa, 4991	>4	>4	>4	>4	>4	>4
Pseudomonas aeruginosa, I-10968	>4	>4	>4	>4	>4	>4
Candida albicans, I-11301	>4	2	4	>4	>4	>4
Candida albicans, I-11134	>4	4	4	>4	>4	>4

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Table 3. Chequerboard titrations of combinations of the peptide amphipathic-2D with different antibiotics

Second antibiotic	Indicator strain	FIC	Effect
Ampicillin	MRSA (LT1338)	0.38	synergy
	MRSA (LT1334)	0.75	no interaction
	Enterococcus faecium (I-11305b)	2.0	no interaction
Oxacillin	MRSA (LT1338)	0.50	synergy
	MRSA (LT1334)	0.56	no interaction
	MRSE (LT1324)	1.0	no interaction
	CoNS (I-10925)	2.0	no interaction
Vancomycin	MRSA (LT1338)	1.0	no interaction
Cefazolin	MRSA (LT1334)	0.38	synergy
	MRSA (LT1338)	0.31	synergy
	E. coli (I-11276b)	1.0	no interaction
	Citrobacter freundii (I-11090)	1.0	no interaction
	CoNS (I-10925)	2.0	no interaction
	MRSE (LT1324)	1.50	no interaction
Ciprofloxacin	MRSA (LT1338)	0.50	synergy
	E. coli (I-11276b)	0.75	no interaction
	Citrobacter freundii (I-11090)	0.63	no interaction
	MRSA (LT1334)	2.0	no interaction
Fosfomycin	MRSE (LT1324)	1.0	no interaction
	MRSA (LT1334)	0.56	no interaction
	MRSA (LT1338)	1.0	no interaction
	CoNS (I-10925)	1.50	no interaction
	E. coli (I-11276b)	2.0	no interaction
	Citrobacter freundii (I-11090)	2.0	no interaction

activity against the enterobacterial strains was somewhat reduced. Generally, the *Stenotrophomonas* and *Pseudomonas* strains as well as *Candida* proved the least susceptible towards this group of diastereomers. On the basis of these activity tests, amphipathic-2D was chosen as a representative peptide for the mode of action studies.

Synergic activities

Chequerboard titrations were carried out using peptide amphipathic-2D in combination with different conventional antibiotics against selected indicator strains (Table 3). In some cases, amphipathic-2D showed some remarkable synergy with β-lactams (ampicillin, oxacillin and cefazolin) against some strains. Synergy was also observed for combinations of amphipathic-2D with the DNA gyrase inhibitor ciprofloxacin. All other FIC indices ranged between >0.5 and ≤4.0 (no interaction); remarkably, no indications of antagonism (FIC of >4.0) were obtained.

Killing kinetics

We selected four species of Gram-positive cocci with comparable *in vitro* susceptibility (MICs of 1 or 2 µM, respectively) and peptide amphipathic-2D for a closer inspection of the killing activity of the diastereomeric peptides. Short-term experiments were carried out to study the immediate impact of the peptides on cells (Figure 1, inserts) and long-term experiments (22 h) for detection of survivors and possible regrowth. The effect of amphipathic-2D was clearly dose-dependent. At 2× MIC, only *E. faecium* showed a continuous decrease in the number of cfu over 22 h (Figure 1b) whereas with all three staphylococcal strains, survival of subpopulations and subsequent regrowth was observed. At 4× MIC, this was still true for the MRSA strain; complete killing was only achieved with *S. simulans* 22. With this strain, we also observed the most immediate impact of the peptide on the cells. At time-point zero, i.e. within seconds after addition of the peptide, the number of cfu was reduced by several log and killing proceeded most rapidly for the first 10 min and then slowed down. The enterococcal strain showed similar killing kinetics. With the MRSA strain, there was no immediate impact of the peptide and the number of cfu was only reduced by less than 2 log within 30 min of incubation. The CoNS strain behaved very differently in that, after rapid reduction in cfu in the first 2 min, cells recovered in the broth culture over the next 20 min before the number of cfu decreased again.

The plating assay used here does not permit the determination of actual time of killing; it may occur during incubation in the broth culture; however, it is equally possible that the cationic peptide does not dissociate from the charged microbial surface and just prevents the formation of a colony on the agar. The latter may be particularly relevant for explaining the immediate response of *S. simulans* 22. Nevertheless, the rapid reduction in the viable count as observed here is a characteristic feature of membrane permeating agents, and it is also characteristic that the overall killing potency of such agents is not very pronounced; membrane permeation can be formed by transient pores and can be overcome by individual cells. In addition, significant portions of a treated population may not be susceptible for physiological reasons. Therefore, biphasic killing curves as observed with *E. faecium*, *S. simulans* and the CoNS strain (Figure 1, inserts) may indicate that, depending on the test strain, an initial membrane-based killing effect may combine with other lethal effects, or even sublethal if acting on their own, that contribute to killing by the AMPs.

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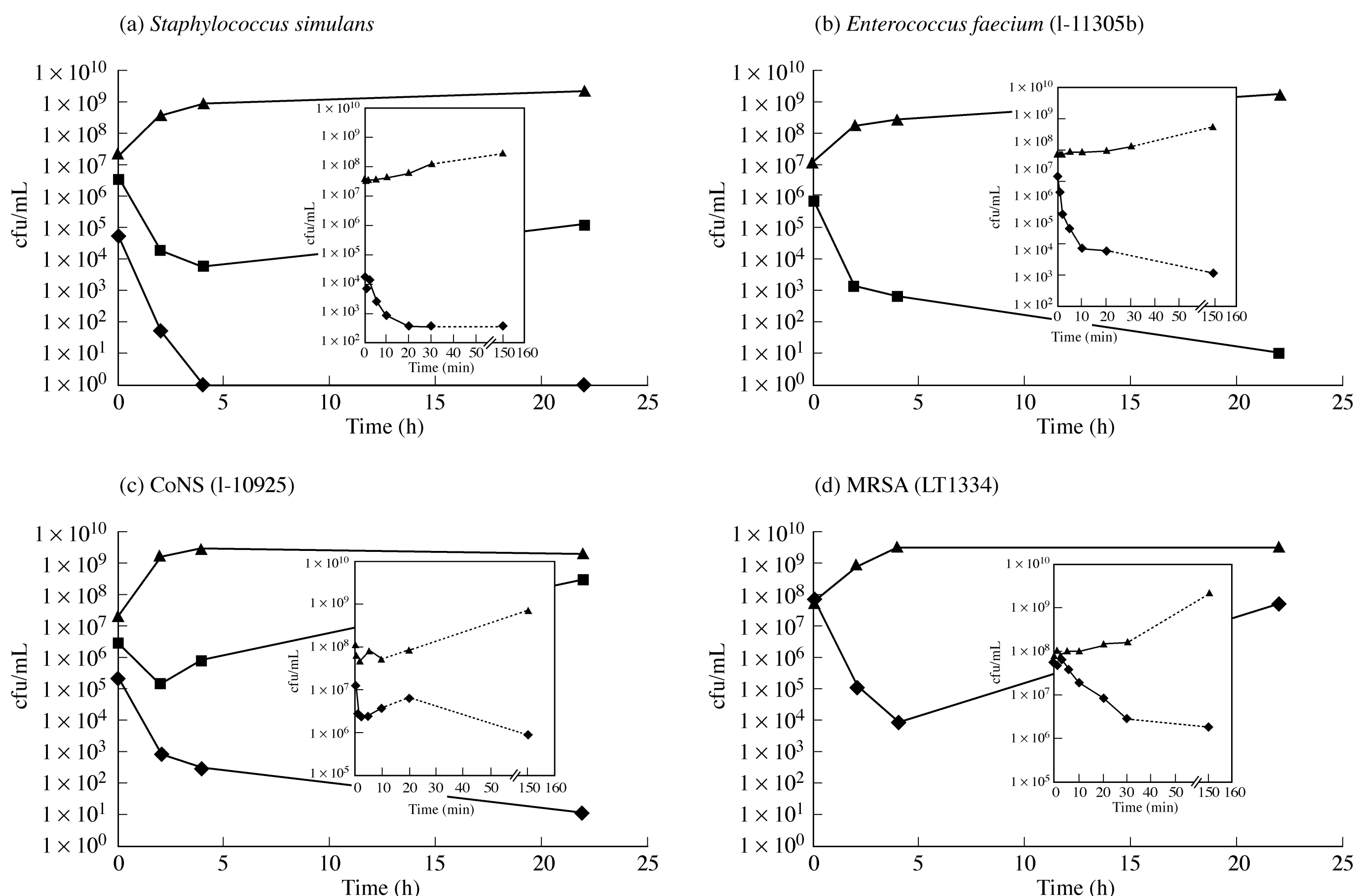

Figure 1. Bactericidal activity of amphiphatic-2D at 2× MIC (filled square) and 4× MIC (filled rhomb) for the respective strain; (filled triangle) control without addition of peptide. Inserts: short-term experiments (0–150 min).

Influence of amphipathic-2D on amino acid accumulation in whole cells

Effects on the integrity of the cytoplasmic membrane should impair energy-dependent transport phenomena. Therefore, we examined the uptake of radioactively labelled glutamate into chloramphenicol-treated Gram-positive bacteria. *S. simulans*, when pretreated with amphipathic-2D, was unable to carry out active transport of glutamate (Figure 2a). The addition of amphipathic-2D to cells which had accumulated glutamate induced a rapid efflux of the marker within a few minutes. In contrast, the clinical isolates were able to transport glutamate in the presence of amphipathic-2D to some extent. With the enterococcal and the MRSA strain, the uptake was approximately 50% of the untreated control. In contrast, the CoNS strain was hardly impaired (Figure 2c); addition of amphipathic-2D to this strain after accumulation of the glutamate induced rapid efflux; however, the amino acid was completely re-imported within a few minutes. No significant efflux was observed with *E. faecium*. When compared with the killing kinetics, the results with *S. simulans* and *S. aureus* agree well: *S. simulans* was killed rapidly and, within the time frame of the uptake experiment, there were not enough viable cells left that could carry out glutamate transport. The MRSA strain was killed more slowly and the remaining viable cells could be responsible for the uptake observed. The efflux kinetics were comparable for both strains. Also, the results with the CoNS strain agreed to some extent since in both assays the strain showed short-term recovery from the action of the peptide. In contrast, the enterococcal cells, 99% of which were inactivated within the first 10 min, retained a substantial capacity for transport, indicating that the actual killing may be delayed until plating.

Effect on the membrane potential of whole cells

The potential across the cytoplasmic membrane ($\Delta\psi$) of bacterial cells can be estimated by measuring the distribution of a radiolabelled, lipophilic cation such as tetraphenylphosphonium bromide (TPP^+). Using this technique, we could show that *S. simulans* 22 maintained a *trans*-negative $\Delta\psi$ of approximately –140 mV, which rapidly decreased after addition of amphipathic-2D; however, $\Delta\psi$ did not drop below –100 mV (Figure 3a). The clinical strains initially showed a $\Delta\psi$ of about –100 to –120 mV (Figure 3b and c). *E. faecium* was only transiently depolarized and $\Delta\psi$ was re-established within about 5 min. $\Delta\psi$ of the CoNS and MRSA strains were hardly affected by the peptide. Clinical strains are known to frequently carry transport proteins of the *S. aureus* QacA-type which export lipophilic drugs and which had also been reported to use the TPP^+ probe as a substrate.[17,18] This was recently noticed with a pair of isogenic strains expressing *qacA* (from plasmid pSK1) and devoid of the plasmid, respectively. In the *qacA*-expressing mutant, $\Delta\psi$ was underestimated by approximately –50 mV (U. Pag, M. Brown, R. H. Skurray, H.-G. Sahl, unpublished data). The presence of such a multidrug transporter leads to underestimation of $\Delta\psi$ in our clinical strains and could explain the relatively low initial potential level observed. Therefore we also used the fluorescent membrane potential probe bis-(1,3-dibutylbarbituric acid) trimethine oxonol [$DiBAC_4(3)$] to monitor the effect of

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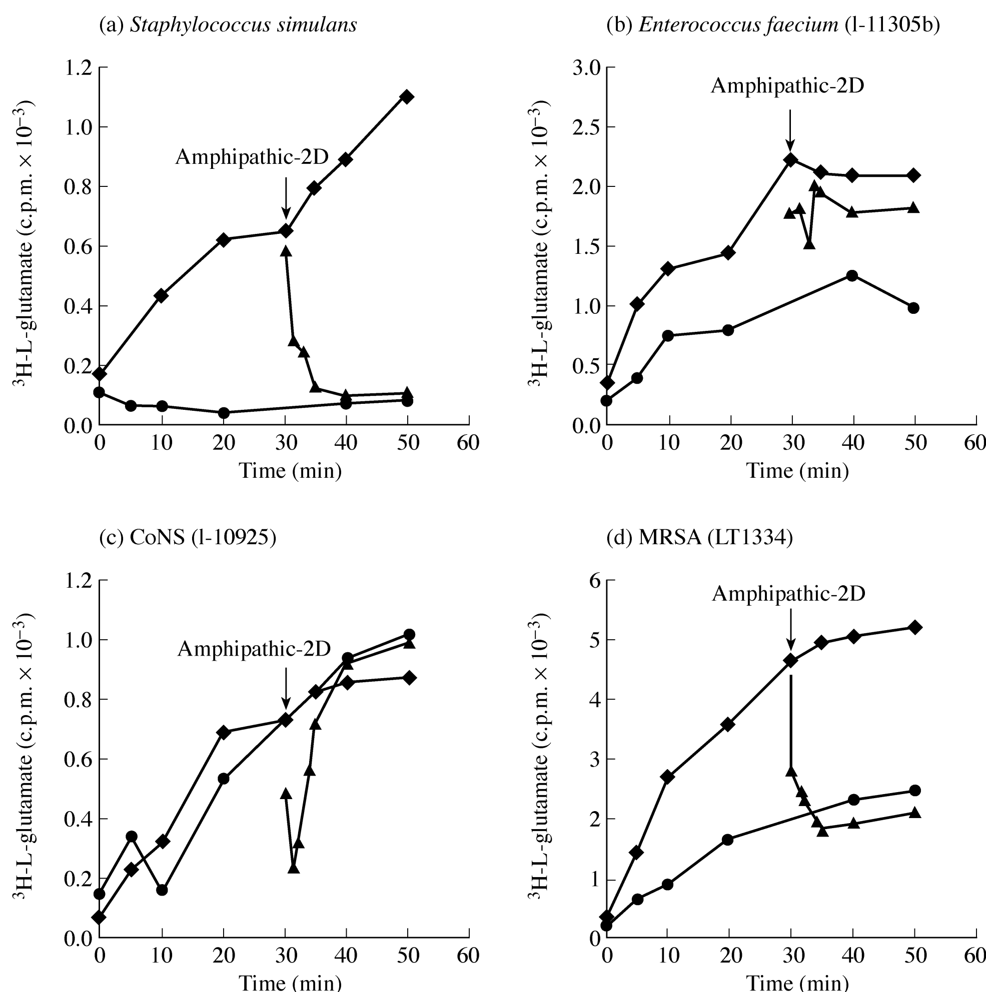

Figure 2. Accumulation of [^{3}H]-L-glutamate into chloramphenicol-treated cells (filled diamonds) and efflux of the radioactive amino acid after addition of amphipathic-2D (10× MIC for the respective strain) (filled triangles); (filled circles) uptake of the radioactive marker by cells pretreated with amphipathic-2D.

amphipathic-2D (Figure 4). Applying this probe, we observed an increase in fluorescence with all strains, being most rapid with *S. simulans* and rather slower with the MRSA strain. Use of fluorescent probes does not allow any quantification of $\Delta\psi$ or changes in $\Delta\psi$; however, since the changes in fluorescence were similar with all peptides, the degree of depolarization may be similar to that measured with TPP^+ and *S. simulans*, i.e. approximately 40 mV. Unlike with TPP^+, depolarization did not seem to be reversible; however, since $DiBAC_4(3)$ is known to traverse depolarized membranes and to subsequently bind to cytoplasmic constituents, its diffusion back across the membrane upon repolarization may be considerably slower than with TPP^+ and therefore this dye may not be suitable for detection of transient effects. In contrast, TPP^+ hardly binds to cellular components and responds quickly to changes in polarization. Taken together, both methods indicate that amphipathic-2D may impair energized bacterial membranes; however, the effect observed on $\Delta\psi$ seems to be too small to fully explain the rapid killing induced by the peptide.

Discussion

Antimicrobial cationic peptides are ubiquitous in nature and offer a new resource for the development of novel anti-infective agents. Although many peptides display a potent and broad-spectrum antimicrobial activity *in vitro*, their potential for applications depends on additional features. As a result of their cationic and amphiphilic properties, the peptides may bind to host components which reduces their bioavailability and generates harmful side effects, such as lysis of red blood cells.[19,20] However, previous studies revealed that incorporation of D-amino acids into various peptides resulted in preservation of antimicrobial activity and reduction in the haemolytic activity.[9,21,22] Furthermore, by site-specific D-amino acid substitution, the sensitivity of the peptides to enzymic degradation can be controlled.[9] Papo *et al.*[13] constructed diastereomers of short model peptides, composed of six lysine and nine leucine residues and one-third of their sequence composed of D-amino acids, which had similar antimicrobial activity to that of their corresponding L-amino acid peptides and which were non-haemolytic. Here, we demonstrated that most of these diastereomers displayed high and broad-spectrum antimicrobial activity against multidrug-resistant clinical isolates of opportunistic and pathogenic organisms. Peptides amphipathic-1D, -2D and -3D especially had a powerful bactericidal effect on most indicator strains including Gram-negative strains as well as fungi. Furthermore, the ability of the peptides to synergize with conventional antibiotics and their possible mode of action was studied.

Synergy with conventional antibiotics

Combination studies showed that amphipathic-2D may act synergically with or add positively to the activity of conventional antibiotics

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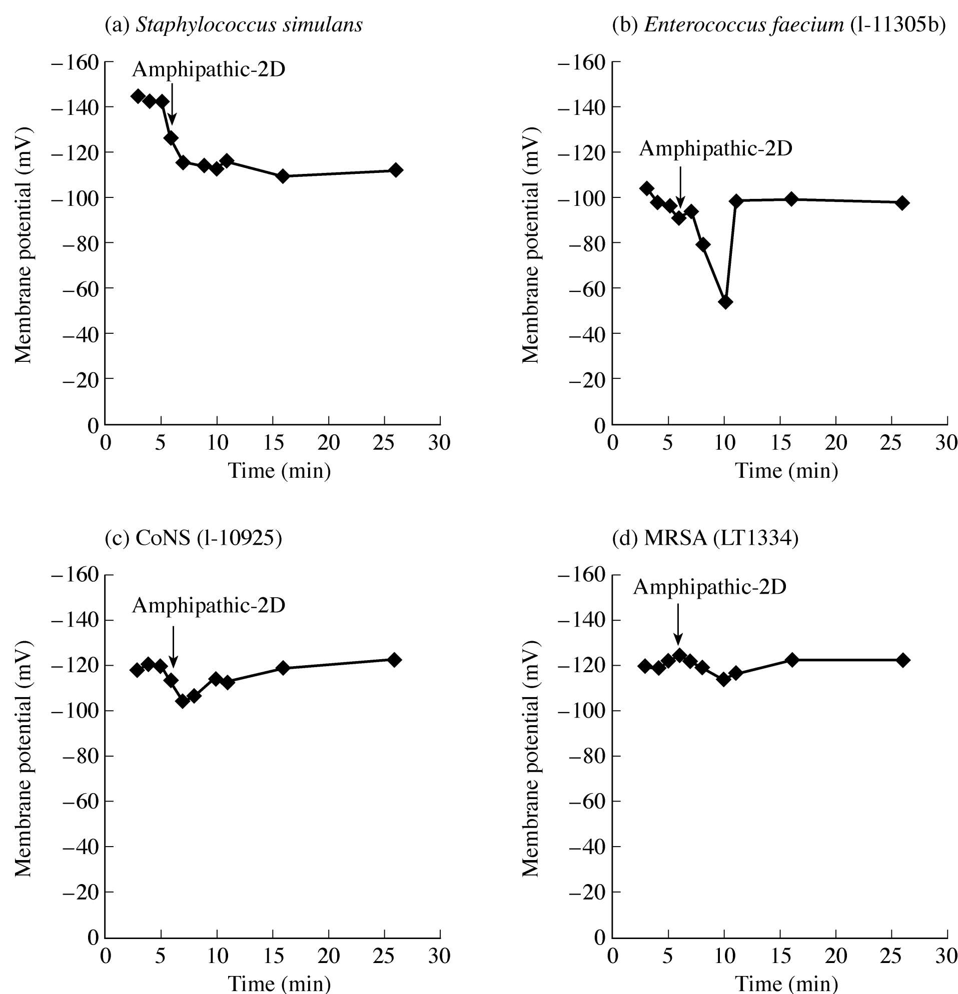

Figure 3. Influence of amphipathic-2D on the membrane potential of different strains. Amphipathic-2D was applied at a concentration of 10 times the MIC for the respective strain.

such as β-lactams and fluoroquinolones. However, in most cases, the positive interactions were restricted to a particular test strain and a general rule for a given species or combination could not be detected. The mechanism of the synergic effect remains largely unknown; we observed both amphipathic-2D showing improved activity while the activity of the conventional antibiotic remained unchanged and *vice versa*; in a few cases, both compounds increased in activity when combined. Thus, through the activity of cell wall biosynthesis inhibitors, the wall architecture may be disturbed and access of amphipathic-2D to the cytoplasmic membrane may be increased, whereas membrane disorganization by the peptide may improve the activity of β-lactams or help gyrase inhibitors to cross the cytoplasmic membrane. Recently, synergy between cationic peptides (buforin II, magainin II or cecropin P1) and β-lactam antibiotics was also shown for Gram-negative species such as *Stenotrophomonas, Acinetobacter* or *Pseudomonas*.[23–25] Amphiphilic cationic peptides may also cause degradation of the peptidoglycan and enhance the activity of the β-lactam antibiotics, by triggering the activity of bacterial murein hydrolases.[26] Furthermore, cationic peptides may compete with divalent cations for binding to lipopolysaccharide (LPS), the major component of the outer membrane of Gram-negative bacteria, which results in permeabilization of the outer membrane and promoted access to sites of peptidoglycan synthesis.[27] Fosfomycin has been reported to synergize with a number of antibiotics;[28,29] however, no synergic effects were observed in combination with amphipathic-2D. In conducting chequerboard titrations, one should recall that such titration assays can only reflect the effectiveness of a combination at a single time point (18 h incubation), but cannot provide information on transient interactions occurring throughout the incubation time.[30]

Mode of action studies

It is widely accepted that the primary target of cationic AMPs is the cytoplasmic membrane; however, the exact mechanism by which these peptides kill bacteria is still not fully understood. Recent studies on the interaction of the diastereomers with model membranes suggested that the antimicrobial activity is based on membrane perturbation as described by the carpet mechanism.[13] The antibiotic peptides are proposed to bind to the surface of the membrane, cover the membrane in a 'carpet-like' manner and disturb its barrier function.[12] To test this model with living microbes, we evaluated data from killing curves, efflux experiments and membrane potential, all obtained under identical experimental conditions with various Gram-positive cocci. Treatment of *S. simulans* 22, a standard laboratory strain which had been used frequently to study the mode of action

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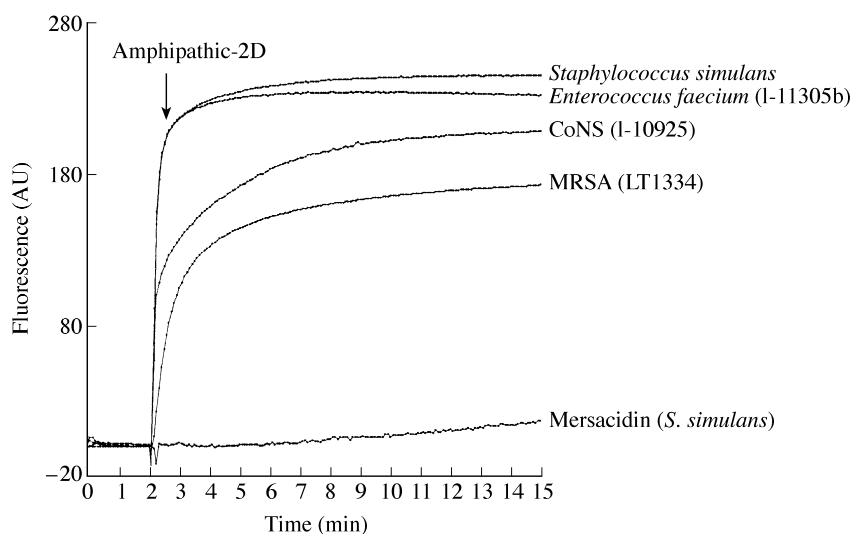

Figure 4. Influence of amphipathic-2D on the membrane potential of different strains probed by $DiBAC_4(3)$ fluorescence. Amphipathic-2D was added at a concentration of 10 times the MIC for the respective strain. Mersacidin was used a negative control; it blocks peptidoglycan biosynthesis and was shown not to influence membrane integrity.[32] The increase in fluorescence upon the addition of peptide was expressed in arbitrary units (AU).

of antibiotic peptides,[16,31,32] yielded a fairly defined picture. We observed rapid killing, significant membrane depolarization and immediate cessation of the uptake as well as rapid efflux of radiolabelled amino acids. Such results are typically seen with membrane permeating agents and are consistent with the data described by Papo *et al.*[13] for spheroplasts of Gram-negative bacteria and intact Gram-positive bacteria.

However, with the clinical isolates, the picture proved more complicated. Killing kinetics were considerably slower and biphasic. Membrane functions, either measured directly as uptake and retention of amino acids or via the membrane potential, appeared to be only partially or transiently impaired; still, the clinical isolates when subjected to standard MIC determination were of comparable susceptibility. In previous studies, it had also been observed that cationic AMPs may vary greatly in their ability to depolarize the cytoplasmic membrane potential of *E. coli* and that there is no direct correlation between the ability to permeabilize the membrane and the antimicrobial activity.[33]

Recently, there has been an increasing number of reports on potential intracellular targets for amphiphilic peptides such as buforin II, indolicidin, cecropin PR-39, HNP-1 and tPMP-1[34–37] and several synthetic peptides.[38,39] Membrane permeabilization is thought to provide access to the cytoplasm in addition to contributing to killing. The data presented in our study are in line with such views. The effects on membrane functions produced by 10× MIC in this study could be clearly demonstrated; however, they appear insufficient to explain the killing kinetics observed and the significant variation in the response of the four test strains, three of which are closely related staphylococci. In addition to the individual properties of the cytoplasmic membrane itself that may influence the susceptibility to cationic peptides,[39] differences in the accessibility of the membrane caused by variations in the molecular set-up of the cell envelope of individual strains may help explain the differences shown for the various clinical isolates. Moreover, not only intracellular targets and processes may suffer from the action of the peptides. *S. simulans* with its unique cell wall lytic system, has been shown to be particularly susceptible to cationic agents that replace the lytic enzymes and prematurely induce cell separation and lysis of the septum. This effect significantly contributes to the killing efficiency of the lantibiotics nisin and Pep5[40,41] and may explain the fast and complete killing of this strain observed with amphipathic-2D. In a more general sense, it had been proposed that depolarization of staphylococcal cells would induce autolysis as a mechanism of programmed cell death[42,43] (reviewed by Bayles[44]). Thus, various degrees of membrane damage and different patterns of additional lethal or sublethal effects produced by the peptides may define the susceptibility of an individual strain and could also explain why results for a given combination of amphipathic-2D with a conventional antibiotic ranged from indifference to synergy within strains of the same species.

The fact that the membrane is a universal target that cannot be changed in its principal set-up, is widely used to explain the broad-spectrum activity of AMPs and their conservation throughout evolution as an effective means for the control of microbes. In light of the additional targets and activities identified recently, it seems justified to broaden this view. The cationic amphiphilic nature of AMPs seems perfectly designed to enable a maximum of interaction, hydrophobic as well as hydrophilic, with cellular constituents. Thus, whenever present in significant concentrations, AMPs could interfere with numerous physiological processes and cause damage at many sites in a cell, which makes development of resistance difficult. Indeed, whereas bacteria exposed to innate immunity factors in the course of evolution have clearly become less susceptible to such peptides,[45,46] they did not develop high-level resistance mechanisms as observed with many widely used conventional antibiotics. In that respect, the diastereomers studied here offer a new resource for the development of novel anti-infective agents either to be used on their own or in combination with established agents to restore and boost their activity.

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