In vitro activity of novel in silico-developed antimicrobial peptides against a panel of bacterial pathogens

A. A. Romani, M. C. Baroni, S. Taddei, F. Ghidini, P. Sansoni, S. Caviran and C. S. Cabassi

Antimicrobial-peptide-based therapies could represent a reliable alternative to overcome antibiotic resistance, as they offer potential advantages such as rapid microbicidal activity and multiple activities against a broad spectrum of bacterial pathogens.

Three synthetic antimicrobial peptides (AMPs), AMP72, AMP126, and also AMP2041, designed by using ad hoc screening software developed in house, were synthesized and tested against nine reference strains. The peptides showed a partial β-sheet structure in 10-mM phosphate buffer. Low cytolytic activity towards both human cell lines (epithelial, endothelial, and fibroblast) and sheep erythrocytes was observed for all peptides. The antimicrobial activity was dose dependent with a minimum bactericidal concentration (MBC) ranging from 0.17 to 10.12 μM (0.4–18.5 μg/ml) for Gram-negative and 0.94 to 20.65 μM (1.72-46.5 μg/ml) for Gram-positive bacteria. Interestingly, in high-salt environment, the antibacterial activity was generally maintained for Gram-negative bacteria. All peptides achieved complete bacterial killing in 20 min or less against Gram-negative bacteria. A linear time-dependent membrane permeabilization was observed for the tested peptides at 12.5 μg/ml. In a medium containing Mg2+ and Ca2+, the peptide combination with EDTA restores the antimicrobial activity particularly for AMP2041. Moreover, in combination with anti-infective agents (quinolones or aminoglycosides) known to bind divalent cation, AMP126 and AMP2041 showed additive activity in comparison with colistin.

Our results suggest the following: (i) there is excellent activity against Gram-negative bacteria, (ii) there is low cytolytic activity, (iii) the presence of a chelating agent restores the antimicrobial activity in a medium containing Mg2+ and Ca2+, and (iv) the MBC value of the combination AMPs–conventional antibiotics was lower than the MBC of single agents alone.

Keywords: antimicrobial peptides; antimicrobial activity; cytoplasmic membrane permeabilization; hemolysis; combined therapy

Introduction

Antimicrobial resistance (AMR) is a major global public health threat. The soaring treatment and social costs associated with this clinical burden are estimated at around €1.5bn per year in Europe [1]. Currently, the establishment of antibiotic resistance is faster than the development of new classes of antibiotics [2]. Despite the recognized need for new antimicrobials for clinical use, only two new classes of antibiotics have been licensed in the last 30 years [3]. Infections attributable to resistant bacteria are becoming even more difficult to treat because of limited therapeutic options. Methicillin-resistant Staphylococcus aureus (MRSA) remains a major threat worldwide, being the most important cause of antibiotic-resistant infections worldwide. Recent trends about MRSA in Europe showed a prevalence above 25% in more than one quarter of European Union countries [4].

A review of reports on multidrug-resistant and extensively drug-resistant Pseudomonas aeruginosa infections revealed resistance to aminoglycosides, antipseudomonal penicillins, cephalosporins, carbapenems, and fluoroquinolones [5]. A major issue for the discovery of new agents against Gram-negative pathogens is their inherent low permeability coupled with multiple mechanisms for rapid efflux of agents out of the cell [6]. For this reason, high doses of drugs need to be administered to achieve effective intracellular concentrations, thus leaving a very low therapeutic range where efficacy can be achieved in the absence of toxicity.

Antimicrobial peptide (AMP)-based therapies have a high potential to become valuable antimicrobial weapons in the next 10 years because they offer potential broad-spectrum activity, rapid bactericidal activity, and low propensity for resistance development [7].

The term ‘antimicrobial peptide’ refers to oligopeptides or polypeptides resulting from the cleavage of larger proteins or nonribosomally synthesized peptides, isolated from organisms of all kingdoms or derived from them [8]. Most AMPs are cationic molecules with spatially separated hydrophobic and charged regions [9]. They likely exert their permeabilizing activity in the bacterial membrane by forming pores or otherwise destabilizing...
or thinning the membrane bilayer. Another mechanism of action, referred to some AMPs, involves the inhibition of the molecular biosynthesis and/or interaction with specific cytoplasmic components of bacteria [10].

AMPs have been hailed as a potential solution to the dearth of novel antibiotic development to reduce the rate of emergence of resistant microbes, as selective pressure is deviated away from one specific molecular target [11].

The aim of this study is focused on the evaluation of antimicrobial activity of novel cyclic AMPs towards a panel of reference bacterial strains. Moreover, the potential synergistic effect of conventional antibacterial drugs and AMPs has been investigated as an intriguing approach to lower the concentration of antibiotics.

Materials and Methods

Pepettes

The peptides composed of 17 residues were designed by using a proprietary screening software developed in house and synthesized from Selleckchem (Houston, TX, USA). Information about purity (>90%) and concentrations was provided by the manufacturer. The freeze-dried peptides were dissolved in phosphate buffer (PB, 10 mM, 0.8709 g/l K_2 HPO_4, 0.6804 g/l KH_2 PO_4) at the concentration of 1 mg/ml.

Sequence generation

The random sequences were generated using a pure Perl implementation of the Mersenne Twister algorithm, a 32-bit pseudo-random number generator developed by Makoto and Takuj [12]. Single amino acids were coded as integers between 1 (Ala) and 20 (Tyr) and randomly inserted in every 4th sequence position (1–17). The first-pass filtering unit controls every single residue of the generated sequence, matching it against a position-related amino acid frequency table built on the basis of more than 1000 experimentally verified AMP sequences. The filtered sequences were then passed to the screening unit.

Neural network

The core of this script is represented by a screening unit that uses the response of five artificial neural network (ANN) (developed in C) to pick out potential AMP sequences. To predict AMPs, the computational neural networks used in this software were three-layer (input–hidden–output), fully connected, feedforward networks. The input layer consists of one node for each descriptor (physicochemical or structural parameter) used to describe the environment of the sequence. The output layer has one node generating the estimated potentiality of the sequence considered to be an AMP. Although no theoretical rules have been developed to determine the number of hidden layers (the number of hidden neurons), the rule of thumb suggests that the number of neurons, sufficient to ensure that the data are adequately represented, should range from half to twice the input nodes used.

Different types of activation functions were used: the identity, sine, hyperbolic tangent, sigmoid, and logistic functions for the hidden layer and the hyperbolic tangent, sigmoid, logistic, and softmax functions for the output layer. The weights of connections between the neurons were initially assigned to uniform random values by using the Levenberg–Marquardt back-propagation method [13].

The total dataset was split into three partitions, and the cases (sequences) were randomly assigned to one of these partitions: (i) the training dataset, used to build the neural network; (ii) the test dataset, smaller than the training dataset, used to verify the network training and to avoid the so-called training overfit; (iii) the validation dataset, used as an independent control of the neural network. This dataset contains the 20% of the total cases randomly chosen. Indeed, the errors for this dataset provide a reliable estimation of the predictive ability of the ANN as it is not used to generate the model. Hence, it is reasonable to assume that the network generalizes well on unseen data when the performance of the network is consistently good on both the test and validation datasets.

Receiver operating characteristic (ROC) curves were plotted for validation and testing databases. Accuracy is measured by the area under the ROC curve. We applied a rough guide for classifying the accuracy of a test by using the traditional academic point system: 0.90–1.0 = excellent, 0.80–0.90 = good, 0.70–0.80 = fair; 0.60–0.70 = poor; 0.50–0.60 = fail.

Homology modeling

Sequence alignment of the three AMPs against templates protegrin-1, arenicin-1, and thanatin-1 was performed with MAFFT [14]. These templates were chosen for their β-sheet folding, in particular arenicin-1 for the similar position of cysteines within sequences. Homology modeling was performed using MODELLER 10v1 [15]. The coordinate file from PDB (1PG1: protegrin-1; 8TFV: thanatin-1; 2JSB: arenicin-1) was used as such; alignment files were prepared from the MAFFT output, and python scripts were written to predict 10 models. The structures were minimized by using Gromacs v4.5.5 [16]. All 30 minimized models (10 per sequence) were evaluated by Procheck [17] performing full geometric analysis with a resolution of 1.5 Å. Ramachandran plot statistics was used to evaluate the best model. The root mean square deviation values were calculated using the CE software [18] by fitting the α-carbon backbone of the predicted model onto the template structures.

CD measurements

Circular dichroism (CD) spectra were recorded on a JASCO model J-715 spectropolarimeter (JASCO, Tokyo, Japan) with a cell path length of 1 mm. The spectra were recorded at 25 °C in the range 180–270 at a scan rate of 10 nm/min. Peptide solutions at 50 μM (100 μg/ml) were prepared by dissolving the peptides in 10 mM PB. Three experiments for each sample were performed and averaged. The data noise was smoothed using the fast Fourier transform noise reduction routine, allowing a decrement of noisy spectra without distorting their peak shapes. The secondary structure content was determined by using the software SOMCD [19]. This software uses the neural network algorithm to estimate the secondary structure composition of protein from CD data.

Bacterial Strains

The following bacterial reference strains, purchased from Mast Diagnostic (Germany) were used for the study:

Preparation of Bacterial Suspension

The bacterial suspension was standardized following the Clinical and Laboratory Standards Institute guidelines [20]. Briefly, the log-growing phase was reached by incubating each strain in brain–heart infusion broth (Difco, USA) at 37 °C in a shaker at 225 rpm for 3–4 h. After being pelleted at 1000 g for 20 min, the bacterial suspension was adjusted spectrophotometrically at 600 nm to an optical density value in the range 0.08–0.13, containing approximately $10^8$ colony-forming unit (CFU)/ml in PB. Fifty microliters of bacterial suspension containing $10^6$ CFU/ml was inoculated into each well, to obtain a final concentration of approximately $5 \times 10^5$ CFU/ml.

Preparation of the Plates and MBC Evaluation

Fifty microliters of peptide, 100–0.17 μM (200–0.4 μg/ml), was added to each well containing 50 μl of bacterial suspension and incubated for 2 h at 37 °C. Then, 20 μl of each dilution was plated onto adequate agar media, incubated for 24 h at 37 °C for the CFU count. The minimum bactericidal concentration (MBC) was considered as the lowest concentration that killed 100% of the bacteria. Moreover, the activity of peptides has been investigated in different NaCl concentrations (10, 50, 125, and 250 mM).

Time-kill Assay

Each standardized bacterial suspension was exposed to peptides at the MBC value and incubated at 37 °C. Aliquots of 20 μl were withdrawn at fixed intervals and spread onto adequate agar plates to obtain viable colonies. After overnight incubation at 37 °C, the CFU were counted. Controls were run without peptide and in the presence of the peptide solvent.

Permeabilization Assay

To assess the ability of peptides to alter the permeability of bacterial membranes, the engineered *E. coli* ML-35 pYC was used [21]. The bacteria were sown in 15 ml Mueller Hinton broth (Difco) with 50 μg/ml ampicillin and left overnight at 37 °C. Three hundred microliters of bacterial suspension was added to 15 ml fresh Mueller Hinton broth with 50 μg/ml ampicillin and incubated for 2–3 h at 37 °C. The suspension was centrifuged at 1000 g for 10 min and the pellet resuspended in PB. A fresh solution of 1.5 mM and 15 mM of CENTA (a beta-lactamase-labile, chromogenic cephalosporin for outer membrane assay) and ONPG (ortho-Nitrophenyl-β-galactoside, chromogenic substrate for inner membrane assay) respectively, was prepared in PB. Sixty microliters of the ONPG or CENTA solution was added to 60 μl of bacterial suspension and placed in a cuvette containing 480 μl of PB. Subsequently, the peptide was added to a final concentration of 5 μM, and the kinetic of color formation was followed for at least 120 min at 405 nm (CENTA) and 600 nm (ONPG).

Hemolysis Assay

Freshly heparinized sheep blood was centrifuged at 100 g for 15 min. The erythrocytes were washed three times with phosphate-buffered saline (PBS), centrifuged at 1000 g for 10 min, and resuspended to a concentration of 2% (v/v) in PB with 308 mM of sucrose to maintain cell osmolarity. Fifty microliters of erythrocyte suspension was incubated with 50 μl of AMPs (final concentrations of 500, 250, 100, 12.5, and 1 μg/ml) for 1 h at 37 °C. Then, the suspension was centrifuged at 1000 g for 5 min, and 150 μl of the supernatants was transferred to a flat-bottomed 96-well plate for spectrophotometric evaluation (450 nm) of hemoglobin release. Negative (0% hemoglobin release) and positive (100% hemoglobin release) controls were obtained by using PB + 308 mM sucrose and 1% Tween-20, respectively.

The percentage of intact erythrocytes was calculated as

$$\left(1 - \frac{A_{\text{pep}} - A_{\text{PB}}}{A_{\text{TWEEN}} - A_{\text{PB}}} \right) \times 100$$

where $A_{\text{pep}}$ was the absorbance at 450 nm in wells containing the peptide, $A_{\text{PB}}$ the absorbance at 450 nm in wells containing buffer, and $A_{\text{TWEEN}}$ the absorbance at 450 nm in the wells containing Tween-20.

In Vitro Cytotoxicity

The cytotoxicity assay was performed using human diploid fibroblasts (cell strain WI-38) derived from female fetal lung, human endothelial cells derived from fetal umbilical vessels (HUVEC), and human lung adenocarcinoma (A549). Cell lines were provided by the American Type Culture Collection (Rockville, MD, USA) and obtained through the Istituto Zooprofilattico Sperimentale della Lombardia e dell’Emilia Romagna (Brescia, Italy). Cultures were maintained in complete medium (Dulbecco’s modified Eagle medium containing antibiotics and 10% fetal calf serum). HUVECs were cultured in complete medium supplemented with 50 μg/ml of endothelial cell growth factor. All cultures were kept in an incubator at 37 °C in a water-saturated atmosphere with 5% CO₂. Routine subcultivations were carried out every week. When cells reached confluence, they were harvested by treatment with 0.25% trypsin in PBS at pH 7.5, counted in a Brucker hemocytometer and plated at a density of $1 \times 10^4$ cells/cm².

For the experiments, the cells were detached from the bottom of the vessels using a trypsin solution, centrifuged, and diluted to a concentration of 25 000 cells/ml. The cells were seeded into 96-well plates at 5000 cells per well in 0.2 ml of media and incubated under standard conditions for 48 h. Subsequently, all the media were removed, and 0.1 ml of fresh media containing a final concentration of 100 μg/ml of peptides was added to the cells. After a 120-min incubation of the cells with the peptides, the quantity of viable cells was determined using the crystal violet method.

Crystal Violet Assay

Cell numbers were evaluated by crystal violet staining as described in [22]. The cells were fixed with 1% formaldehyde and stained with 0.1% crystal violet in PBS. The unbound dye was removed by washing with water. Bound crystal violet was solubilized with 0.2% Triton X-100 in PBS. Light extinction that increases linearly with the cell number was analyzed at 570 nm.

Combination Study

A combination study was performed by using the broth checkerboard synergy test. A series of twofold dilutions of AMP126, AMP2041, or colistin were tested in combination with concentrations of twofold dilutions of amikacin, tobramycin, and levofloxacin. The concentrations used ranged from one quarter to fourfold the MBC of a given antibiotic against *P.*
The result of the checkerboard test was expressed as the fractional inhibitory concentration (FIC) index calculated using the following formula:

\[
FIC \text{ index} = \frac{\text{MBC drug A in combination}}{\text{MBC drug A alone}} + \frac{\text{MBC drug B in combination}}{\text{MBC drug B alone}}
\]

The method of the lowest FIC index [23] was used for the interpretation of results. Synergy was defined as an FIC index of ≤0.5, additivity as an FIC index greater than 0.5 and lower than 1.0, indifference as an FIC index range of 1–4, and antagonism as an FIC index of >4.0.

### Table 1. Neural networks performances and characteristics

<table>
<thead>
<tr>
<th>ID</th>
<th>Network name</th>
<th>Training performance (%)</th>
<th>Test performance (%)</th>
<th>Validation performance (%)</th>
<th>Error function</th>
<th>Hidden activation</th>
<th>Output activation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ANN 21–23–2</td>
<td>100</td>
<td>98.2</td>
<td>97.5</td>
<td>Entropy</td>
<td>Tanh</td>
<td>Softmax</td>
</tr>
<tr>
<td>3</td>
<td>ANN 21–26–2</td>
<td>100</td>
<td>97.2</td>
<td>96.5</td>
<td>Entropy</td>
<td>Tanh</td>
<td>Softmax</td>
</tr>
<tr>
<td>11</td>
<td>ANN 21–32–2</td>
<td>100</td>
<td>98.2</td>
<td>97.4</td>
<td>Entropy</td>
<td>Logistic</td>
<td>Softmax</td>
</tr>
<tr>
<td>12</td>
<td>ANN 21–37–2</td>
<td>100</td>
<td>97.7</td>
<td>97.2</td>
<td>Entropy</td>
<td>Tanh</td>
<td>Softmax</td>
</tr>
<tr>
<td>14</td>
<td>ANN 21–42–2</td>
<td>100</td>
<td>98.3</td>
<td>97.1</td>
<td>Entropy</td>
<td>Tanh</td>
<td>Softmax</td>
</tr>
</tbody>
</table>

ANN 21–23–2: 21 input layers, 23 hidden layers, and two output layers. See text for further details.

**Figure 1.** ROC plots. ROC curves and the performance level that could be expected from random guessing. The greater the area that lies below the ROC curve, the greater the power of the classifier. Area under the curve for ANNs (average): 0.986; area under the curve for random guessing: 0.50.

**Results**

**Peptides**

An ad hoc software was developed by one of the authors (AAR) to mine putative AMPs. The script consists of four main blocks: the generation unit (random amino acid sequence generation), the filtering unit (first-pass sequence filtering), the screening unit (prediction of potential antimicrobial activity), and the scoring unit (second-pass sequence filtering). The core of this script is represented by the screening unit that uses the response of five neural networks (developed in C) to pick out potential AMP sequences.

To highlight the most representative properties of AMP, we have selected 21 physicochemical and structural parameters such...
as isoelectric point, amphipathicity, lipophilicity, charge, Boman index, flexibility, helix, or β-sheet propensity. These descriptors were evaluated against a dataset of antimicrobial and non-AMPs to investigate their reliability to act as classifiers. The ANN system has been developed to classify peptides into two groups: AMP and non-AMP. To build the AMP training dataset, several databases such as AMSDb [24], APD2 [25], CAMP [26], and Defensins Knowledgebase [27], containing AMP records, were used, whereas the UNIPROT database was used to construct the non-AMP dataset. After peptide-containing non-standard amino acids or peptides with sequences not fully characterized were removed, the total dataset contained 4043 peptides, of which 1000 were AMPs and 3000 were non-AMPs. The ANN system was trained using 2100 peptides. To establish the optimal size of the hidden layer (the number of the neurons), the system started with 11 neurons in the hidden layer, and the network performance improved with increasing neurons. The standard error of the estimate was used as a criterion for the selection of the optimum number of hidden neurons. The best-performing networks had 23, 26, 32, 37, and 42 neurons. Less than 21 neurons did not reach an overall accuracy of 90%, and more than 45 nodes did not improve the results on the test and validation datasets but determined a loss of model generality. The activation functions were hyperbolic tangent and logistic. The softmax function was the output function of all networks (Table 1).

In Figure 1, the ROC showed that the ANNs built were able to correctly classify peptides in the two considered groups (AMP and non-AMP). The overall accuracy of the method was around 95% and is similar in both the validation and testing datasets, meaning that there was no training overfitting. The successful results obtained both for peptide classification may suggest that the set of physicochemical/structural parameters chosen is complete and can appropriately describe the AMPs (Figure 2A). The proportion of potential AMPs mined by the software was around 0.01–0.2%. Three sequences showing the typical fingerprint of AMPs as defined by Yeaman et al. [28] were selected and synthetized. The AMPs showed charges ranging from +5 to +7, a percentage of hydrophobic residues not less than 35%, a polar angle not less than 100° and around 109–112°, and amphipathicity represented by a hydrophobic moment around +4 (Table 2).

The structural characteristics of peptides were inferred from a computational model built by homology modeling on that of arenicin-1 (Figure 2B). They were constituted by two-stranded antiparallel β-sheet stabilized by three up to six interstrand hydrogen bonds and one disulfide bond between Cys3 and Cys16. The strands of the β-sheet were connected by a type I β-turn. The charge of peptides ranged from +5 (AMP72) to +7 (AMP2041). The electrostatic potential of the representative peptide (AMP126) revealed a diffuse positively charged surface, with large hydrophobic areas separated by the positively charged arginine and lysine side chains. This distribution of charged and hydrophobic areas was determined by a right-handed twist of the β-sheet structure.

**CD Spectra**

The secondary structures of AMP72, AMP126, and AMP2041 were estimated using a spectropolarimeter. The CD spectra were baseline corrected and smoothed by the fast Fourier noise reduction algorithm. The CD spectrum of AMP72, AMP126, and AMP2041 determined in 10 mM PB showed contents of α-helix, β-sheet, and random coils of 11–12%, 34–38%, and 38–40%, respectively (Figure 3).

![Figure 2.](image_url) Software flowchart and peptide structure. (A) Ad hoc software developed to mine putative antimicrobial peptides. (B) The structure of the representative peptides AMP126 shows two-stranded antiparallel β-sheets and one disulfide bond (Cys3–Cys16); the electrostatic potential surface of the representative AMPs; the Coulombic–hydrophobic surface, reveals the characteristic distribution of a charged area onto peptide AMP2041.
Antimicrobial Activity

The results shown in Table 3 highlight a similar MBC against *P. aeruginosa* (<5 μg/ml), in particular, AMP126 was the most potent with an MBC value of 0.17 μM. Furthermore, a high activity against *E. coli* was observed, in particular for AMP2041 (0.88 μM) and AMP126 (0.98 μM). Against *S. maltophilia*, only AMP2041 showed an MBC lower than 5 μM. AMP2041 and AMP126 showed an MBC around 5 μM against *S. enteritidis*, whereas AMP72 had an MBC of 1.76 μM. AMP2041 had the highest activity against *S. aureus* (1.83 μM), whereas AMP126 had the lowest (20.65 μM). Only AMP2041 and AMP2041 showed high activities against the methicillin-resistant variant (0.94 and 1.11 μM, respectively). AMP126 only showed a potent activity against *S. agalactiae* (1.24 μM).

In an effort to understand the influence of monovalent ions on antimicrobial peptide activity, the antibacterial activity was investigated in different salt concentrations. As shown in Table 4, the activity was generally maintained for Gram-negative bacteria. At the highest NaCl concentration used (250 mM), AMP2041 achieved the best performance with a 90% reduction of CFU count at 12.5 μg/ml. At the same NaCl concentration, AMP126 still showed a good antimicrobial activity (85% at 12.5 μg/ml), whereas AMP72 showed an activity of 80.5% and 64.2% at 100 and 12.5 μg/ml, respectively. A low antibacterial activity against *S. aureus* beyond 125 mM NaCl was observed (<15% vs control).

Permeabilization Assay

The loss of cytoplasmic membrane integrity has been investigated by evaluating the release of periplasmic β-lactamase and cytoplasmic β-galactosidase in engineered *E. coli*. In Figure 4A, the profile of outer membrane permeabilization (CENTA assay) has been reported. As clearly shown in Figure 4A, within the first minute, a fast permeabilization/damage of outer membrane occurred. The AMPs AMP2041, AMP72, AMP126, and colistin showed a similar time-dependent permeabilization rate. This profile was clearly different from those obtained using EDTA (5 mM) and amikacin. Similarly, the permeabilization of inner membrane (ONPG assay) increased linearly in time (Figure 4B). The behavior of colistin was quite different, as clearly shown in Figure 4B. Indeed, the incubation of *E. coli* ML-35pYC with colistin failed to determine an increase of optical density in the first 30 min, after which a moderate increase was observed.

**Time Course of AMP Activity towards Gram-positive and Gram-negative Strains**

To evaluate the effect of peptides over time, cultures of *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, and *S. aureus* ATCC 25923, representative of Gram-negative and Gram-positive species, were incubated with 12.5 μg/ml of AMPs. Figure 5 shows that AMP126 and AMP2041 exerted strong antimicrobial activity against *P. aeruginosa* with a significant reduction of 50% in CFU count within 10 min after peptide incubation, with a complete

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**Table 2.** Physicochemical properties

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>Experimental mass (calculateda)</th>
<th>Charge</th>
<th>Hydrophobic ratio</th>
<th>Mean hydrophobicityb</th>
<th>Amphipathicity</th>
<th>Lipophilicity</th>
<th>Boman indexc</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMP72</td>
<td>KGCAVKVGLTIXVCK</td>
<td>1814.34 (1816.40)</td>
<td>5</td>
<td>52.0%</td>
<td>–1.32</td>
<td>2.11</td>
<td>0.70</td>
<td>0.38</td>
</tr>
<tr>
<td>AMP126</td>
<td>KTCRQWWRGKFIKCV</td>
<td>2249.50 (2251.48)</td>
<td>6</td>
<td>52.0%</td>
<td>–0.92</td>
<td>2.04</td>
<td>3.18</td>
<td>1.81</td>
</tr>
<tr>
<td>AMP2041</td>
<td>HKCAIKWKGVHVKYCA</td>
<td>2025.48 (2027.50)</td>
<td>7</td>
<td>47.0%</td>
<td>–2.90</td>
<td>3.30</td>
<td>0.29</td>
<td>1.42</td>
</tr>
</tbody>
</table>

aTheoretical mass peptide.
bThe hydrophobicity values were calculated by using the combined consensus hydrophobicity scale of Tossi et al. [21].
cThe Boman index is defined as the sum of the free energies of the respective side chains for the transfer of cyclohexane to water [40].

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**Figure 3.** CD spectra of AMP72, AMP126, and AMP2041. The CD spectrum was recorded in 10 mM PB, and the structural content of AMPs was estimated by the SOMCD software.
killing after 20 min. For AMP72, the reduction of 50% CFU occurred 30 min after peptide addition, with complete killing in 90 min.

The count of *E. coli* was reduced by 50% within the first 5 min for AMP126 whereas it needed 20 min for AMP2041. It is interesting to note that AMP72 exhibits a bacteriostatic effect during the first 30 min with a fast bactericidal phase in the next 20 min and a complete killing of bacteria in 50 min after peptide incubation.

The type of activity against *S. aureus* was similar for all tested peptides. As shown in Figure 5, there was a reduction of 50% in CFU count in 60 min, which reached 66% within 120 min, and failure to exert a complete killing of the tested bacteria.

**Cytolytic Activity**

It is well known that several AMPs exhibit cytolysis activities. To evaluate the cytotoxicity of AMPs against eukaryotic cell membrane, we firstly examined their hemolytic activity against sheep red blood cells using different concentrations (1, 12.5, 100, 250, and 500 μg/ml). After 1 h of incubation, negligible hemolytic activity (<1%) was observed in control wells as well as at 1 and 12.5 μg/ml (<2%). Low hemolytic activity compared with controls (not greater than 6%) was observed at 100 μg/ml for all the tested peptides. At the highest peptide concentration used, the hemolysis remained lower than 15% for all peptides (Table 5). Furthermore, the toxic effect of peptides on endothelial, epithelial, and mesenchymal cell lines was evaluated. The overall results indicate a lytic activity ranging from 2% to 6% against all the tested cell lines (Table 6). In particular, AMP72 showed the lowest overall toxicity (3%), followed by AMP126 (4%) whereas AMP126 showed the lowest toxicity (2%) against endothelial cells (HUVEC) and the highest toxicity was observed for AMP2041 (6%); this latter result seems to match the data observed in the hemolysis assay.

**EDTA Assay**

The antimicrobial activity of majority of the AMPs is more susceptible to the presence of divalent ions; therefore, molecules able to bind divalent cations could be effectively employed in enhancing the bactericidal activity of them. In Table 7, the activity of AMP126 and AMP2041 in the presence of different concentrations of EDTA is shown.

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**Table 3.** Antimicrobial activity of AMPs against a panel of Gram-negative and Gram-positive bacteria

<table>
<thead>
<tr>
<th>Peptides</th>
<th>Bacterial strains</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>Pseudomonas aeruginosa</em></td>
</tr>
<tr>
<td>AMP2041</td>
<td>2.14 (4.35)</td>
</tr>
<tr>
<td>AMP72</td>
<td>1.81 (3.30)</td>
</tr>
<tr>
<td>AMP126</td>
<td>0.17 (0.40)</td>
</tr>
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</table>

Table 4. Effect of NaCl on the antimicrobial activity of AMPs

<table>
<thead>
<tr>
<th>Peptide</th>
<th>NaCl (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 g/ml (%)</td>
</tr>
<tr>
<td></td>
<td>10 g/ml (%)</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em> ATCC 27853</td>
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</tr>
<tr>
<td>AMP72</td>
<td>&lt;10</td>
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<tr>
<td>AMP126</td>
<td>&lt;10</td>
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<td>AMP2041</td>
<td>&lt;10</td>
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<td><em>Escherichia coli</em> ATCC 25922</td>
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<td><em>Staphylococcus aureus</em> ATCC 25923</td>
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<td>AMP72</td>
<td>&lt;10</td>
</tr>
<tr>
<td>AMP126</td>
<td>&lt;10</td>
</tr>
<tr>
<td>AMP2041</td>
<td>&lt;10</td>
</tr>
</tbody>
</table>

The bacteria were pre-incubated to the mid-logarithmic phase in brain–heart infusion broth (Difco) and adjusted to 10^8 CFU/ml. Fifty microliters of bacterial suspension were incubated with 50 μl of PB containing different concentrations of peptides. MBC was considered as the lowest concentration that killed 100% of the bacteria. Three independent experiments were performed in duplicate.

NA, defined not active when the AMP concentration was equal or greater than 100 μg/ml (55 μM).
Intriguing to speculate whether chinolones or aminoglycosides, which both interact with divalent ions similarly to EDTA, may affect, enhancing or reducing, the antimicrobial activity of AMPs. The MICs of amikacin, tobramycin, colistin, and levofloxacin (0.25–4 μg/ml) against the reference strain of *P. aeruginosa* were within the acceptable MIC range indicated by EUCAST (2012). The MICs were used to calculate the range of concentrations tested in the combination study (Table 8). On the basis of activity in the high-salt environment, the peptides enrolled in the combination study were AMP126 and AMP2041. A significant increase in the activity of colistin in the combination regimen (an FIC value of 1.05–1.50) was observed (Table 9). Notably, peptide-containing regimens showed an interesting additive activity. In particular, a bactericidal combination of 0.2 μg/ml AMP126 with 1 μg/ml amikacin gave an FIC of 0.63, indicating additivity. The combination between AMP126 (0.2 μg/ml) and levofloxacin (2 μg/ml) gave an FIC index of 0.51, close to the upper breakpoint value for synergy. Similar results were obtained for AMP2041 (0.2 μg/ml) and levofloxacin (2 μg/ml), with an FIC index of 0.52. On the contrary, no changes were noted when peptides were associated with tobramycin (FIC index value = 1.125 vs 1.50), compared with colistin.

**Discussion**

The continuous emergence of a clinical bacterial strain resistant to one or more different conventional antibiotic drugs has potentially become in the last decade a major medical and veterinary problem throwing us into the so-called post-antibiotic era [29]. This trend has prompted the search for novel anti-infective agents, among which AMPs achieved increasing attention [30]. Therefore, novel AMPs have been developed by using an *in silico* approach.

The secondary structure of AMP72, AMP126, and AMP2041 was investigated to verify the amphiphatic structure. A similar CD spectrum was obtained for all AMPs in 10 mM PB, with a 30–33% β-sheet content. This result suggests that AMPs did not show a predominant conformation in hydrophilic environments in agreement with literature data [31,32].

The peptides showed excellent activity against Gram-negative bacteria whereas good activity against Gram-positive bacteria was observed. In particular, AMP126 was most powerful against *P. aeruginosa* with an MBC of 0.17 μM and the worst against Gram-positive bacteria with the exception of *S. agalactiae* (MBC value = 1.24 μM). AMP2041 and AMP72 killed MRSA to an extent similar to that of the nonresistant strain, indicating that the mechanisms of methicillin resistance did not affect the peptides, further evidence that the mechanism of action of these peptides is likely very different from that of conventional antibiotics.

Bacterial-killing kinetic assays against *P. aeruginosa* and *E. coli* revealed that AMP2041 and AMP126 achieved complete bacterial killing in 20 min or less. These results establish a potential application of AMP2041 and AMP126 in the treatment of bacterial infection. Previous studies suggested that the activity of cationic peptides relies on their ability to contact the anionic pathogen outer membrane via electrostatic interaction and subsequently infiltrate the membrane via hydrophobic interaction [33]. This suggests that besides the structural determinants, it is important to have a positive charge density and optimal balance between hydrophilic and hydrophobic peptide surfaces. Moreover, the amphiphilic conformation is thought to be responsible for the ability to kill bacterial cells by disrupting bacterial membranes.

**Combination Study**

The therapeutic potential for AMPs relates to their use as primary agents or to reconstitute/amplify the efficacies of conventional antibiotics. On the basis of the results obtained with EDTA, it was
The target of many cationic peptides is the bacterial membrane, resulting in cell death likely due to loss of membrane integrity. This has been demonstrated for a range of cationic peptides [28]. The results presented about AMP72, AMP126, and AMP2041 showed different permeabilization kinetics, and as shown in Table 3, there was no obvious correlation between the kinetics of membrane permeabilization and antimicrobial activity. In particular, killing curves carried out in conjunction with the permeabilization assay indicated that similar cytoplasmic membrane permeabilization profiles did not correspond with similar killing rates, as clearly shown for the peptides AMP72, AMP126, and AMP2041 (Figures 4 and 5); for example, a significant reduction in the numbers of bacteria (90–99%) appeared to occur within the first few minutes after addition of the peptide AMP126 (Figure 4), at which point the permeabilization of the cytoplasmic membrane was not complete and was only approximately 25% of the maximum permeabilization possibly occurring with AMP2041 and AMP72 (Figure 5).

The passage of the peptide through the membranes is expected to cause an increase in membrane permeability, and this might account for the lag time between depolarization and killing. This lag time should be explained by taking into account that ion leakage subsequent to peptide permeabilization is likely due to the formation of small pores that cannot allow the spillage of enzymes such as β-lactamase or β-galactosidase out of bacteria [34,35]. Membrane permeabilization occurring after cell death may be a secondary or subsidiary effect of the peptides.

Salt inactivation of antibacterial activity is dependent on the concentration of NaCl. Whereas AMP2041 and AMP126 exhibit salt-sensitive activities similar against Gram-negative bacteria, the loss of antibacterial activity observed in the presence of high NaCl concentrations was observed in AMP72. It is conceivable that the higher net positive charge in AMP2041 as compared with that in AMP72 may have a role in modulating antibacterial activity in the presence of NaCl. It is possible, also, that the distribution of positive charges may be a determinant of salt sensitivity of antibacterial peptides. Moreover, this salt-dependent antibacterial activity might be explained by taking into account the membrane damage induced by these peptides. The results presented about AMP72, AMP126, and AMP2041 showed different permeabilization kinetics, and as shown in Table 3, there was no obvious correlation between the kinetics of membrane permeabilization and antimicrobial activity. In particular, killing curves carried out in conjunction with the permeabilization assay indicated that similar cytoplasmic membrane permeabilization profiles did not correspond with similar killing rates, as clearly shown for the peptides AMP72, AMP126, and AMP2041 (Figures 4 and 5); for example, a significant reduction in the numbers of bacteria (90–99%) appeared to occur within the first few minutes after addition of the peptide AMP126 (Figure 4), at which point the permeabilization of the cytoplasmic membrane was not complete and was only approximately 25% of the maximum permeabilization possibly occurring with AMP2041 and AMP72 (Figure 5).

Table 5. Haemolysis assay

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>Haemolysis (% vs control)</th>
<th>Peptide</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>P72</td>
</tr>
<tr>
<td>500</td>
<td>12.0</td>
<td>12.5</td>
</tr>
<tr>
<td>250</td>
<td>4.2</td>
<td>6.9</td>
</tr>
<tr>
<td>100</td>
<td>1.5</td>
<td>5.30</td>
</tr>
<tr>
<td>12.5</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>1</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

The sheep erythrocytes were incubated with AMPs (100, 12.5, and 1 µg/ml) for 1 h at 37 °C. The supernatant was spectrophotometrically evaluated at 450 nm for hemoglobin release. Negative (0% hemoglobin release) and positive controls (100% hemoglobin release) were obtained by using PB + 308 mM sucrose and 1% Tween-20, respectively.

Table 6. Cytotoxicity assay

<table>
<thead>
<tr>
<th>Human cell lines</th>
<th>Cytotoxicity (% vs control)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Peptide (100 µg/ml)</td>
</tr>
<tr>
<td></td>
<td>P72</td>
</tr>
<tr>
<td>HUVEC (endothelial)</td>
<td>3</td>
</tr>
<tr>
<td>WI-38 (fibroblast)</td>
<td>4</td>
</tr>
<tr>
<td>AS49 (epithelial, adenocarcinoma lung)</td>
<td>2</td>
</tr>
</tbody>
</table>

The human cell lines were incubated with AMPs (100 µg/ml) for 2 h at 37 °C, and the quantity of viable cells was spectrophotometrically evaluated at 570 nm by using the crystal violet method.

The results presented about AMP72, AMP126, and AMP2041 showed different permeabilization kinetics, and as shown in Table 3, there was no obvious correlation between the kinetics of membrane permeabilization and antimicrobial activity. In particular, killing curves carried out in conjunction with the permeabilization assay indicated that similar cytoplasmic membrane permeabilization profiles did not correspond with similar killing rates, as clearly shown for the peptides AMP72, AMP126, and AMP2041 (Figures 4 and 5); for example, a significant reduction in the numbers of bacteria (90–99%) appeared to occur within the first few minutes after addition of the peptide AMP126 (Figure 4), at which point the permeabilization of the cytoplasmic membrane was not complete and was only approximately 25% of the maximum permeabilization possibly occurring with AMP2041 and AMP72 (Figure 5).
account that peptides with few hydrogen bonds between β-sheet strands suffer high ionic strength. High-salt environment weakened the initial electrostatic interactions between the peptides and bacterial targets, thereby reducing their bactericidal activities as suggested by Zasloff [11].

In the present study, the bactericidal and cytolytic activities were compared using the same incubation time and the same buffer (Tables 5 and 6). Assuming that both bacterial killing and hemolysis were the final consequence of peptide binding, the hemolysis assay showed that tested peptides were safe to eukaryotic cells. Furthermore, the safety of peptides on eukaryotic cells was investigated on human cell lines (epithelial, endothelial, and mesenchymal). The results confirmed, at the highest concentration used, the selectivity of AMP72, AMP126, and AMP2041 towards bacterial membranes.

In the hemolysis assay, the first concentration that determines hemolysis was 100 μg/ml, which translates back to an intravenous dose of 10 mg/kg (a 20-g mouse has a blood volume of approximately 2 ml). It is known that AMPs with no effect whatsoever on red blood cells at 250–500 μg/ml already cause internal bleeding at 20–50 mg/kg dose in vivo.

It is important to stress that the information derived from in vitro hemolysis or cytotoxicity assays should be considered carefully in representing AMP-selective toxicity because it may not realistically reflect the potential cytotoxicity in vivo [28].

It is well known, at least for Gram-negative bacteria, that the integrity of the outer membrane lies in the divalent cations binding to lipopolysaccharide (LPS) [36]. In the case of Gram-negative bacteria, AMPs interact with the lipid A moiety of LPS by determining a disorganization of the outer membrane through displacement of divalent cations and consequently disruption of the outer membrane bilayer. The majority of AMPs were more susceptible to the presence of Mg²⁺ ions, owing to a higher affinity of Mg²⁺ to LPS. Therefore, molecules or agents able to bind divalent cations could be effectively employed in enhancing the destabilization of bacterial surface [37]. It has been shown that conventional antibiotics such as quinolones chelate divalent cations with a mechanism similar to that of EDTA [38]. Furthermore, the antagonism of aminoglycoside absorption by divalent cations for P. aeruginosa has been demonstrated [39].

The proposed chelation of divalent cations by quinolones does not match the results obtained with colistin and hence does not confirm such activity. On the contrary, the combination of levofloxacin and AMP126 or AMP2041 may improve or increase the access of conventional antibiotic or peptides or both to the intracellular target.

<table>
<thead>
<tr>
<th>Peptide (μg/ml)</th>
<th>Pseudomonas aeruginosa ATCC 27853</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMP2041</td>
<td></td>
</tr>
<tr>
<td>200</td>
<td>0</td>
</tr>
<tr>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td>25</td>
<td>0</td>
</tr>
<tr>
<td>12.5</td>
<td>0</td>
</tr>
<tr>
<td>6.25</td>
<td>0</td>
</tr>
<tr>
<td>3.2</td>
<td>47</td>
</tr>
<tr>
<td>1.6</td>
<td>90</td>
</tr>
<tr>
<td>Control</td>
<td>400</td>
</tr>
<tr>
<td>AMP126</td>
<td></td>
</tr>
<tr>
<td>200</td>
<td>0</td>
</tr>
<tr>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td>25</td>
<td>0</td>
</tr>
<tr>
<td>12.5</td>
<td>0</td>
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<tr>
<td>6.25</td>
<td>0</td>
</tr>
<tr>
<td>3.2</td>
<td>0</td>
</tr>
<tr>
<td>1.6</td>
<td>6</td>
</tr>
<tr>
<td>Control</td>
<td>400</td>
</tr>
</tbody>
</table>

Bacteria were incubated with twofold dilutions of peptides in PB containing 2.4 mmol/l Ca²⁺ and 0.84 mmol/l Mg²⁺, in the presence of different concentrations of EDTA. Values represent CFU count; controls were determined for each EDTA concentration. Italic bold characters indicate MBC.

### Table 7. Effect of EDTA on the antimicrobial activity of AMPs

<table>
<thead>
<tr>
<th>Peptide (μg/ml)</th>
<th>PB (10 mM), without Mg²⁺ and Ca²⁺</th>
<th>PB (10 mM) with Mg²⁺ and Ca²⁺ + EDTA (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMP2041</td>
<td>200</td>
<td>0</td>
</tr>
<tr>
<td>AMP126</td>
<td>200</td>
<td>0</td>
</tr>
<tr>
<td>100</td>
<td>0</td>
<td>300</td>
</tr>
<tr>
<td>50</td>
<td>0</td>
<td>350</td>
</tr>
<tr>
<td>25</td>
<td>0</td>
<td>400</td>
</tr>
<tr>
<td>12.5</td>
<td>0</td>
<td>400</td>
</tr>
<tr>
<td>6.25</td>
<td>0</td>
<td>400</td>
</tr>
<tr>
<td>3.2</td>
<td>47</td>
<td>400</td>
</tr>
<tr>
<td>1.6</td>
<td>90</td>
<td>400</td>
</tr>
<tr>
<td>Control</td>
<td>400</td>
<td>400</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Peptide (μg/ml)</th>
<th>Acceptable MIC range (μg/ml)</th>
<th>Tested range (μg/ml)</th>
<th>MIC (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amikacin</td>
<td>1-4</td>
<td>0.125-8</td>
<td>1</td>
</tr>
<tr>
<td>Colistin</td>
<td>0.25-2</td>
<td>0.06-16</td>
<td>1-2</td>
</tr>
<tr>
<td>Levofloxacin</td>
<td>0.5-4</td>
<td>0.125-8</td>
<td>2-4</td>
</tr>
<tr>
<td>Tobramycin</td>
<td>0.25-1</td>
<td>0.064-256</td>
<td>0.25</td>
</tr>
</tbody>
</table>

*Acceptable range as indicated in EUCAST (2012).
However, we cannot entirely exclude the hypothesis that the increased/improved uptake of drugs could be the result of sequential inhibition in the cell wall protein biosynthesis.

Interestingly, the results obtained by combining AMPs with amikacin indicate an additive effect and could be not entirely explained by increased permeability of the outer membrane alone. In general, no synergy was detected when combining our peptides with aminoglycosides or quinolones, antibiotics usually excluded by LPS binding. Hence, a general permeability-increasing effect on the LPS by the peptides may not be adequate to allow for a synergic interaction to be revealed.

Conclusions

AMP72, AMP126, and AMP2041 proved the following: (i) there is excellent activity against Gram-negative bacteria, (ii) there is low cytolytic activity, (iii) the presence of a chelating agent restores the antimicrobial activity in a medium containing Mg²⁺ and Ca²⁺, and (iv) the MBC value of the combination AMPs-conventional antibiotics was lower than the MBC of single agents alone.

Therefore, the data so far obtained strongly suggest the potential of AMP72, AMP126, and AMP2041 to be considered as antifective agents. We are also aware that our study has the limitations of an in vitro study and does not allow us to draw definitive conclusions (or to predict) about the in vivo efficacy or toxicity. However, our results represent an intriguing starting point to perform the in vivo experiments to estimate the usefulness of these peptides in light of a potential clinical use.

Acknowledgement

We thank Dr. Lisa Elviri, Dr. Annalisa Bianchera, and Fabio Pollorsi for their help in technical assistance in circular dichroism assay.

References


