Investigations into the killing activity of an antimicrobial peptide active against extensively antibiotic-resistant K. pneumoniae and P. aeruginosa

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Abstract

SET-M33 is a multimeric antimicrobial peptide active against Gram-negative bacteria in vitro and in vivo. Insights into its killing mechanism could elucidate correlations with selectivity.

SET-M33 showed concentration-dependent bactericidal activity against colistin-susceptible and resistant isolates of P. aeruginosa and K. pneumoniae. Scanning and transmission microscopy studies showed that SET-M33 generated cell blisters, blebs, membrane stacks and deep craters in K. pneumoniae and P. aeruginosa cells. NMR analysis and CD spectra in the presence of sodium dodecyl sulfate micelles showed a transition from an unstructured state to a stable α-helix, driving the peptide to arrange itself on the surface of micelles.

SET-M33 kills Gram-negative bacteria after an initial interaction with bacterial LPS. The molecule becomes then embedded in the outer membrane surface, thereby impairing cell function. This activity of SET-M33, in contrast to other similar antimicrobial peptides such as colistin, does not generate resistant mutants after 24 h of exposure, non-specific interactions or toxicity against eukaryotic cell membranes, suggesting that SET-M33 is a promising new option for the treatment of Gram-negative antibiotic-resistant infections.

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1. Introduction

Extensive use of broad-spectrum antibiotics has led to the development and spread of extensively antibiotic-resistant strains of bacteria, making antimicrobial resistance a global problem. Antibiotic-resistant bacteria kill 25,000 people in the EU every year. Infections such as urinary tract infections [1], pneumonia [2] and septicemia [3] are increasingly associated with multi-drug resistant Gram-negative bacteria in all regions of the world [4]. These antibiotic-resistant bacteria generally cause infections associated with increased risk of poor clinical outcome and mortality compared to non-resistant strains of the same bacteria [5]. The World Health Organization warns that antimicrobial resistance is an increasingly serious threat to global public health and calls for action across all government sectors and society [6] to avoid a dreaded “post-antibiotic” era.

A recent rekindling of antibiotic research has shown that the antimicrobial peptide class of antibiotics is particularly promising for use against infections caused by multidrug-resistant microorganisms [7–9]. Antimicrobial peptides (AMPs) are an important component of the natural defenses of most living organisms, and >2500 AMPs have been registered in the Antimicrobial Peptide Database (http://aps.unmc.edu/AP/main.php) [10]. However, despite their desirable characteristics, antimicrobial peptides have had limited pharmaceutical development due to their toxicity, instability and manufacturing costs, and therefore only a few AMPs have actually been approved for clinical use [11–12].

The most common mechanism of antimicrobial killing by antimicrobial peptides is disruption of the cytoplasmic membrane, for example by pore formation, which is rather non-specific but highly efficient [13–15]. Alternative mechanisms of action include peptide translocation into the cytoplasm where the antibiotic interferes with bacterial metabolic processes, such as protein synthesis or DNA replication, while...
other peptides are known to interact directly with specific membrane components [16–18]. In particular, cationic AMPs have been demonstrated to induce anionic lipid clustering which appears to arrest bacterial growth or trigger cell death [19–20].

SET-M33 is an antimicrobial peptide that has been extensively studied in recent years [21–25]. It is a cationic non-natural peptide built in a branched form (Fig. 1) that makes it more resistant to degradation in biological fluids [26]. SET-M33 has shown efficacy against a number of Gram-negative multi-drug and extensively drug-resistant isolates [21,24]. It has also shown acceptable toxicity in human cells and in mice [22], as well as anti-inflammatory activity [23]. Nevertheless, its mechanism of action has not yet been researched in depth. In this study we used different techniques to study the mechanism of action of SET-M33 against two Gram-negative bacterial species, K. pneumoniae and P. aeruginosa: 1) bacterial killing kinetics over time to see concentration-dependency and onset of resistance; 2) electron microscopy imaging of treated bacteria to visualize membrane disturbance; 3) hemolytic activity to assess eukaryotic cell toxicity and 4) NMR and circular dichroism to study the structure of the peptides in the presence of micelles and their ability to stabilize in a facial amphiphilic helix.

2. Materials and methods

2.1. Peptide synthesis

All peptides were prepared by solid-phase synthesis through standard Fmoc chemistry using a Syro multiple peptide synthesizer (MultiSynTech, Witten, Germany). Side chain protecting groups were 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl for R, t-butoxycarbonyl for K and t-butyl for S (Iris Biotech GmbH, Marktredwitz, Germany). The final products were cleaved from the solid support, de-protected by treatment with TFA containing triisopropylsilane and water (95:2.5:2.5), and precipitated with diethyl ether. Final peptide purity and identity was confirmed by reverse-phase chromatography on a Phenomenex Jupiter C18 analytical column (300 Å, 250 × 4.6 mm) by mass spectrometry.

Q-33 (linear peptide, QKKIRVRLSA) was produced on TentaGel S RAM resin (Iris Biotech GmbH, Marktredwitz, Germany). The crude peptide, released as amide, was purified by reverse-phase chromatography on a Phenomenex Jupiter C18 column (300 Å, 250 × 10 mm) in a linear gradient, using 0.1% TFA/water as eluent A and acetonitrile as eluent B (from 99% to 50% of A in 30 min). The compound was characterized on a MALDI-TOF mass spectrometer (Ultraflex III Bruker Daltonics): QKKIRVRLSA-NH₂ MALDI-MS: 4682.48 [M + H]+; RP-HPLC: t_R = 19.62 min, purity > 99%.

SET-M33 (tetrabranched peptide, (KKIRVRLSA)₄K₂K₂) was synthesized on a Fmoc4-Lys2-Lys-QKKIRVRLSA-NH₂, MALDI-MS: 1198.97 [M + H]+; RP-HPLC: t_R = 21.10 min, purity > 99%. SET-M33 solubility, water ≥ 20 mg/mL, saline ≥ 20 mg/mL, PBS ≥ 15 mg/mL.

2.2. Selection of colistin-resistant mutants of K. pneumoniae R-DYK 4861 and P. aeruginosa B-162

K. pneumoniae R-DYK 4861 and P. aeruginosa B-162 were both extensively antibiotic-resistant clinical isolates and colistin-susceptible. Colistin-resistant mutants of both strains were obtained by step-wise serial passages of bacterial strains [27] in BBL™ Mueller Hinton II (MH-II) broth (Becton, Dickinson Benelux N.V., Erembodegem, Belgium) containing colistin concentrations in the range 2–64 mg/L (colistin sulfate, Sigma-Aldrich Chemie BV, Zwijndrecht, the Netherlands). This involved sub-culturing 100 µL of overnight culture of the bacteria (grown in MH-II broth) into MH-II broth containing 2 mg/L colistin and incubating overnight at 35 °C. In subsequent steps 100 µL of overnight culture was sub-cultured into MH-II broth containing twofold increases in the concentration of colistin until the highest colistin concentration of 64 mg/L was achieved. Finally, 100 µL volumes were sub-cultured onto solid MH-II medium (Becton, Dickinson Benelux N.V., Erembodegem, Belgium). Colistin-resistant colonies were then characterized phenotypically using VITEK® 2.

2.3. Phenotypic characterization of bacterial isolates

Phenotypic characterization of K. pneumoniae B-DYK 4861 and P. aeruginosa B-162 isolates and their colistin-resistant mutants was performed by determining their susceptibility for a panel of 18 different antibiotics from the main classes of antibiotics using the VITEK® 2 anti-microbial identification system (BioMérieux Benelux BV, Zaltbommel, The Netherlands) and AST-N140 cards (Vitek AMS). Interpretation of antimicrobial susceptibility was based on EUCAST 2017 guidelines [28] (Table 1S Supplementary data).

2.4. Antimicrobial susceptibility of bacterial isolates – MIC assay

Antimicrobial susceptibility was assessed by determining the Minimum Inhibitory Concentration (MIC) of SET-M33 and colistin using the broth microdilution technique according to 2017 EUCAST guidelines. The MIC assay measures visible inhibition of bacterial growth after 24 h exposure of bacteria to antibiotic in MH-II broth. The two-fold antibiotic concentration range used for SET-M33 and colistin was 0.063–64 mg/L.

2.5. Concentration- and time-dependent bactericidal activity of SET-M33 and colistin – TKK assay

The concentration- and time-dependent killing capacity of SET-M33 was determined using time-kill kinetic (TKK) assays, as previously described [29]. Briefly, stationary-phase MH-II broth cultures of K. pneumoniae R-DYK 4861 and P. aeruginosa B-162 were diluted in 25 mL MH-II broth until a density of approximately 7 × 10⁵ colony forming units (CFU/mL) was achieved. Bacterial cultures were exposed to antimicrobial antibiotics at 2-fold increasing concentrations for 24 h
at 37 °C under shaking conditions at 96 rpm at 37 °C. Next, 1 mL samples were taken at 0, 1, 2, 4, 6 and 24 h of antibiotic exposure and centrifuged at 12500 × g for 5 min to pellet the cells, which were then resuspended in sterile PBS. Agar plates were incubated for 24 h (K. pneumoniae) or 48 h (P. aeruginosa) at 37 °C in order to determine the number of CFU. The lower limit of quantification in this assay was 5 CFU/mL (log 0.7).

For colistin-susceptible bacterial isolates, the two-fold antibiotic concentration range was 0.125–64 mg/L for SET-M33 and colistin alike.

For colistin-resistant isolates, the two-fold antibiotic concentration range was 0.125–64 mg/L for SET-M33 and 1–512 mg/L for colistin.

Plates showing regrowth of bacteria after 24 h antibiotic exposure were examined for changes in antibiotic susceptibility using the antibiotic MIC assay described above, but with an antibiotic concentration range of 0.5–512 mg/L for the colistin-resistant isolates.

2.6. Sample preparation for electron microscopy

P. aeruginosa PAO1 and K. pneumoniae ATCC 13833 cells in logarithmic phase were resuspended at 2 × 10^8 CFU/mL in PBS and incubated with 1.5 μM SET-M33 at room temperature for 15, 30 and 60 min. The mixture was then centrifuged for 5 min at 10000 r.p.m.

2.7. Scanning electron microscopy

Centrifuged bacteria were resuspended in 500 μL PBS and a drop of liquid cell suspension was placed on untreated glass coverslip for five minutes. The coverslip was then fixed for immersion in 2.5% glutaraldehyde solution in phosphate buffer 0.1 M pH 7.2 (PB) for 2 h at 4 °C, washed in PB, postfixed in 1% OsO4 in PB for 30 min at 4 °C, dehydrated in an ascending alcohol series, and dried in a Balzers CPD 030 CO2 critical point dryer.

The coverslip was then mounted on an aluminum stub, coated with 20 nm gold in a Balzers MED010 sputtering device, and observed in a Philips XL20 scanning electron microscope with an electron accelerating voltage of 20 kV.

2.8. Transmission electron microscopy

Centrifuged bacteria were fixed in 2.5% glutaraldehyde solution in phosphate buffer 0.1 M pH 7.2 (PB) for 2 h at 4 °C, washed in PB, postfixed in 1% OsO4 in PB for 30 min at 4 °C, dehydrated in an ascending alcohol series, and dried in a Balzers CPD 030 CO2 critical point dryer.

The coverslip was fixed in 1% OsO4 in PB for 30 min at 4 °C, dehydrated in 1% OsO4 in PB for 30 min at 4 °C, dehydrated in an ascending alcohol series, and embedded in epon/araldite resin that was polymerized at 60 °C.

Ultrathin sections (60 nm thick) were cut from samples on a Reichert-Jung Ultracut E ultramicrotome, mounted on 200-mesh copper grids, stained with uranyl acetate and lead citrate and observed in a FEI Tecnai G2 SPIRIT transmission electron microscope using an electron accelerating voltage of 100 kV under standard operating conditions.

2.9. Nuclear magnetic resonance

All NMR samples were prepared by dissolving lyophilized peptides in 500 μL H2O/D2O (95:5) to a final concentration of 1.0 mM, with the exception of SET-M33, which was dissolved to a final concentration of 0.25 mM. Since the resonance of N-terminus amide would be missed in aqueous media due to chemical exchange, the peptide sequence of exception of SET-M33, which was dissolved to a

3. Results and discussion

The K. pneumoniae and P. aeruginosa isolates used in the following experiments were resistant to a wide range of beta-lactam antibiotics (penicillins, cephalosporins and carbapenems) including the 4th generation cephalosporin cefepime, aminoglycosides (gentamycin and tobramycin) and fluoroquinolones (ciprofloxacin and norfloxacin). Interpretation of antimicrobial susceptibility was based on European Committee on Antimicrobial Susceptibility Testing (EUCAST) 2017 guidelines [28].

3.1. Susceptibility of bacterial isolates to SET-M33 and colistin – minimum inhibitory concentration MIC

The susceptibility of the colistin-susceptible K. pneumoniae B-DYK 4861 and P. aeruginosa B-162 isolates and their colistin-resistant mutants to SET-M33 and colistin in terms of MIC are shown in Table 1.

3.2. SET-M33 and colistin activity against colistin-susceptible and colistin-resistant K. pneumoniae B-DYK 4861 and P. aeruginosa B-162 – time-kill kinetics TTK

The major killing effect of an antibiotic against an organism depends on the exposure time or concentration of the drug at the active target site. In the present study we investigated extensively drug-resistant K.
pseudomonas and P. aeruginosa populations, taking the effects of exposure time and concentration into account.

Colistin-resistant and colistin-resistant K. pneumoniae isolates were killed by ≥99.9% after 2 h of exposure to ≥4 mg/L and ≥8 mg/L of SET-M33, respectively (the first concentration above the grey shade in Fig. 2A, B). This was followed by bacterial re-growth up to the level of non-exposed bacteria after 24 h exposure to ≤4 mg/L and ≤16 mg/L, respectively. Colistin killed ≥99.9% of the colistin-susceptible K. pneumoniae isolate after 2 h of exposure to ≥0.5 mg/L (Fig. 2C) and bacterial re-growth after 24 h exposure was observed up to ≤32 mg/L. In the colistin-resistant isolate, colistin concentration-dependent killing was observed in the first hour of exposure but ≥99.9% killing was not achieved after 2 h of exposure (Fig. 2D).

For P. aeruginosa, ≥99.9% killing was achieved in colistin-susceptible and colistin-resistant isolates after 2 h of exposure to ≥8 mg/L of SET-M33 (Fig. 2E and F).

Colistin killed ≥99.9% of bacteria in the colistin-susceptible P. aeruginosa isolate after 2 h of exposure to a concentration ≥1 mg/L (Fig. 2C). In the colistin-resistant P. aeruginosa isolate, colistin concentration-dependent killing was observed in the first 2 h of exposure, followed by bacterial re-growth up to the level of non-exposed bacteria after 24 h, at all the colistin concentrations tested (Fig. 2H).

We also showed that exposure to SET-M33 sterilized colistin-susceptible K. pneumoniae and P. aeruginosa populations already at one-fold (16 mg/L) (Fig. 2A) and four-fold (64 mg/L) (Fig. 2E) the MICs, respectively. In contrast, after exposure to colistin, sterilization only occurred at the extremely high concentration of 64 mg/L (Fig. 2C and G), which is 128-fold and 32-fold the MICs of colistin-susceptible K. pneumoniae and P. aeruginosa, respectively. Colistin-resistant K. pneumoniae and P. aeruginosa were sterilized with 64 mg/L SET-M33 (Fig. 2B and F) in both cases (four-fold the MIC) but never with colistin (Fig. 2D and H).

SET-M33 and colistin showed concentration-dependent bactericidal activity against colistin-susceptible and colistin-resistant isolates of K. pneumoniae and P. aeruginosa. This result is similar to the action of different widely used antibiotics e.g. aminoglycosides and fluoroquinolones, as well as other AMPs [31].

SET-M33 retards activity against both colistin-susceptible and colistin-resistant bacteria. This result indicates that SET-M33 is impervious to the mechanisms associated with colistin resistance.

### Table 1

<table>
<thead>
<tr>
<th>Bacterial isolate</th>
<th>Colistin-susceptible R-DYK 4861</th>
<th>Colistin-resistant R-DYK 4861</th>
<th>Colistin-susceptible P. aeruginosa B-162</th>
<th>Colistin-resistant P. aeruginosa B-162</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>AMP</strong></td>
<td><strong>MIC</strong> (μM)</td>
<td><strong>Range</strong></td>
<td><strong>MIC</strong> (μM)</td>
<td><strong>Range</strong></td>
</tr>
<tr>
<td>SET-M33</td>
<td>16</td>
<td>16–16</td>
<td>16</td>
<td>16–32</td>
</tr>
<tr>
<td>Colistin</td>
<td>0.50</td>
<td>0.25–0.50</td>
<td>&gt;512</td>
<td>&gt;512</td>
</tr>
</tbody>
</table>

Colistin resistance did not affect SET-M33 susceptibility in these two strains, confirming our previous data on seven different colistin-resistant K. pneumoniae strains [21]. SET-M33 showed a MIC of 8 mg/L for K. pneumoniae ATCC 13833 and P. aeruginosa PA01 [21].

The effect of SET-M33 treatment at MIC (8 mg/L SET-M33) for 15, 30 and 60 min on bacterial cell morphology was studied by scanning electron microscopy (SEM) and transmission electron microscopy (TEM). TEM images showed that after 30 min of SET-M33 treatment, the surfaces of K. pneumoniae ATCC 13833 and P. aeruginosa PA01 cells lost their smoothness and developed super blisters (Fig. 3). After 60 min of SET-M33 treatment, bacterial cells appeared larger than control cells and many were empty and showed large holes, mainly at the poles.

The supramolecular structure of peptides is important and has been extensively studied in interactions of peptides with bacterial
membranes [16,38–39]. The NMR spectra of free SET-M33 showed a chemical shift index typical of a random coiled conformation and no significant inter-residue NOEs, indicating that the peptide explores a large conformational space. On addition of sodium dodecyl sulfate (SDS) micelles, the NMR spectrum of SET-M33 undergoes a generalized broadening, caused by the production of slow-tumbling high-mass species, precluding further analysis. The high-mass species could be aggregates of SET-M33-SDS, possibly promoted by the multicentricity of SET-M33. We therefore synthesized an analogue peptide, Q-33, in linear form and capped it with an additional amino acid (Q-KKIRVRLSA) at the N-terminus in order to generate a model of SET-M33 that could be studied in the presence of SDS. Q-33 has an extremely lower half life in serum and plasma than SET-M33, 2 h versus 24 h, and consistently it has a lower activity against Gram-negative bacteria [40–41]. The additional Q allowed us to study the structure of all residues, including the particular, NOE signals of the Hα-HNi+3,HNi-HNi+2 types for residue i = 1 to 6 are diagnostic of α-helix conformation spanning residues 1–9 (Fig. 5A). Peak integration of a total set of 32 NOEs allowed

Fig. 2. Concentration- and time-dependent bactericidal activity of SET-M33 and colistin against isolates of K. pneumoniae R-DVK 4861 (A-D) and P. aeruginosa B-162 (E-H). Bacterial cultures were exposed to two-fold increasing concentrations of SET-M33 or colistin for 24 h at 37 °C under shaking conditions. Samples were collected at 1, 2, 4, 6 and 24 h, and after centrifuging and washing, were sub-cultured onto antibiotic-free solid media to determine CFU counts after incubation for 24 h at 37 °C. Data are medians of 3 experiments. Grey shading indicates ≥99.9% killing achieved after 2 h of exposure.
us to calculate the relative interproton distances, which were used as constraints for simulation of torsion angle dynamics and subsequent peptide structure calculations. The resulting structure showed a regular α-helix encompassing the full-length of the peptide.

To assess the interaction geometry of the helix with SDS micelles, a paramagnetic solution-NMR experiment was performed. TOCSY (total correlation spectroscopy) spectra of the peptide-micelle system were acquired in the presence and absence of increasing concentrations of the paramagnetic probe Gd(III)(DTPA-BMA). The soluble probe causes nuclear spin relaxation proportional to the local surface accessibility of the molecule investigated [42–44]. Relaxation is measured by calculating the decrease in peak volumes on addition of the probe, and is summarized as a bare number, the attenuation value A, which ranges from 0 to 2 and can be determined for each known proton peak. Protons shielded by contact with the solvent, and therefore not accessible to the probe, show low A values, while exposed protons show high A values (Fig. 5B, upper part). Attenuation analysis was carried out on the Q-33-micelle system and an accurate accessibility profile was determined. Residues I3 and L7 showed much lower accessibility of the probe to their sidechains, and this can be ascribed to shielding by close interaction with the micelle surface. Charged residue pairs K1/R4 and K2/R6, situated on the opposite side of the helix, showed a higher A value, while V5 and S8 showed the highest attenuation due to their high exposure to the solvent. The resulting structure was a helix lying on the micelle surface with I3/L7 sidechains inserted into the SDS assembly, and R and K sidechains arranged parallel to the surface, their charged tips interacting with the negative sulfate groups (Fig. 5B).

Finally, we wanted to assess whether SET-M33 and Q-33 had the same ability to adopt a helix conformation in a membrane mimicking environment, using circular dichroism. The technique proved to be applicable to both the tetrabranched and the linear peptide. SET-M33 and Q-33 CD spectra were recorded at room temperature in water and in SDS 30 mM. In line with the NMR experiments, SET-M33 showed a non-structured conformation in water but the CD spectrum shifted sharply to longer wavelengths in the presence of SDS micelles (Fig. 5C), and the calculated helix ratio switched from 0% to 30%. A similar result was obtained with Q-33 (Fig. 5D): the spectra recorded in water

### Table 2
Change in colistin-susceptible *K. pneumoniae* R-DYK 4861 and *P. aeruginosa* B-162 susceptibility to SET-M33 and colistin after 24 h exposure to antibiotic as determined by MIC assay.

<table>
<thead>
<tr>
<th>Antibiotic concentration (mg/L)</th>
<th>SET-M33 (mg/L)</th>
<th>Colistin (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Colistin-susceptible <em>K. pneumoniae</em></td>
<td>Colistin-susceptible <em>P. aeruginosa</em></td>
</tr>
<tr>
<td>Control</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>0.125</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>0.25</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>0.5</td>
<td>16</td>
<td>&gt;64</td>
</tr>
<tr>
<td>1</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>2</td>
<td>16</td>
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</tr>
<tr>
<td>4</td>
<td>32</td>
<td>16</td>
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<tr>
<td>8</td>
<td>NR</td>
<td>16</td>
</tr>
<tr>
<td>16</td>
<td>NR</td>
<td>12*</td>
</tr>
<tr>
<td>32</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>64</td>
<td>NR</td>
<td>NR</td>
</tr>
</tbody>
</table>

Colistin-susceptible bacterial cultures were exposed to two-fold increasing concentrations of SET-M33 or colistin for 24 h at 37 °C under shaking conditions. When bacteria re-grew after 24 h exposure to antibiotic, their MIC susceptibilities (performed in triplicate) were also determined and the median value reported. NR, no bacterial re-growth.

* One out of three samples did not show bacterial re-growth.
* Two out of three samples did not show bacterial re-growth.

![Fig. 3.](image-url) Scanning electron micrographs (SEM) of *K. pneumoniae* ATCC 13833 and *P. aeruginosa* PAO1. SEM micrographs of A) untreated *K. pneumoniae*; B) *K. pneumoniae* after 30 min incubation with SET-M33 at MIC; C) *K. pneumoniae* after 60 min incubation with SET-M33 at MIC; D) untreated *P. aeruginosa*; E) *P. aeruginosa* after 30 min incubation with SET-M33 at MIC; F) *P. aeruginosa* after 60 min incubation with SET-M33 at MIC. Scale bar 2 μm.
had a calculated helix ratio of 3.3% compared to 39% in the presence of SDS micelles. The CD spectra of SET-M33 and Q-33, recorded with or without micelles, were very similar, confirming that Q-33 was a reliable model of SET-M33 for the NMR experiments.

3.6. Selectivity/hemolytic activity

The ability of cationic peptides to stabilize as an α-helix, and particularly as a facial amphiphilic structure, is often associated with self-aggregation and undesired peptide interactions with blood proteins due to exposed hydrophobic surfaces [45]. The tendency to self-aggregate is correlated to hydrophobicity and provides an indirect estimate of the propensity for partitioning into lipid membranes with unselective detergent-like characteristics. Antimicrobial peptides need to be selective for prokaryotic membranes; a well-established method of verifying their ability to damage eukaryotic cell membranes is to measure their hemolytic activity [48]. Our results showed that red blood cells incubated with increasing concentrations of SET-M33 did not show more than 26% lysis, even using a concentration of 1.8 mg/mL (320 μM), which is >100-fold the MIC (Fig. 6).

In contrast with other cationic peptides [49], SET-M33 showed very little hemolytic activity against red blood cells, although it shares the ability to form a regular α-helix shape. Indeed, SET-M33 also proved not to be cytotoxic to human bronchial epithelial cells (16HBE14 and CFBE41) [23]. Self-aggregation, often associated with amphiphilic structures and undesired secondary effects [49], was also studied in FPLC experiments (Supplementary material – Fig. 1S, A) and showed that no self-aggregation occurred when SET-M33 was dissolved in PBS. The presence of LPS promoted the formation of high-mass hetero-multimers, but not homo-multimers (Supplementary material – Fig. 1S, B), in line with what we observed and described above in relation to SDS.

4. Conclusions

Current understanding of the mechanism of action of cationic AMPs includes a first step of electrostatic attraction between the cationic peptide and the negatively charged bacterial outer membrane. As SET-M33 is a cationic amphiphilic peptide it is attracted to the negatively charged bacterial membrane. Once close to the microbial surface, AMPs need to cross the polysaccharide cell wall barrier before interacting with the...
brane permeability, as observed with cross cell wall and plasma membranes [41], causing enhanced membrane death may realistically be caused by impairment of the membrane host-interactions or toxicity against eukaryotic cells [8]–[49] and indeed correlates with an acceptable tolerability profile obtained in toxicology studies in mice [21].

SET-M33 does not show any cross-resistance with colistin in colistin-resistant bacteria and, differently from colistin, it appears to generate substantially no resistance within 24 h of exposure in extensively resistant isolates of *K. pneumoniae* and *P. aeruginosa*. These observations suggest that the two peptides have a different mechanism of action. SET-M33 is attracted onto the surface of bacteria by the anionic charges of the cell wall. Being the core of the branched peptide completely flexible, we speculate that in the presence of phospholipid-bilayers, the peptide can assume a star like conformation, where hydrophobic residues are buried in the surface and hydrophilic residues point upwards. The disturbance of bilayers integrity, produced by SET-M33’s peculiar way to interact with membranes, makes it more difficult for bacteria to trigger resistance mechanisms than it is for colistin.

Finally SET-M33, in contrast to other similar antimicrobial peptides such as colistin, does not generate resistant mutants after 24 h of exposure, and non-specific interactions or toxicity against eukaryotic cell membranes, suggesting that SET-M33 is a promising new option for the treatment of Gram-negative antibiotic-resistant infections.

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**Transparency declarations**

Chiara Falciani, Alessandro Pini and Luisa Bracci are cofounders of Setlance srl. SET-M33 is owned by Setlance srl.

**Appendix A. Supplementary data**

Supplementary data to this article can be found online at [http://dx.doi.org/10.1016/j.bbamem.2017.06.001](http://dx.doi.org/10.1016/j.bbamem.2017.06.001).

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