Selection and redesign for high selectivity of membrane-active antimicrobial peptides from a dedicated sequence/function database

Tomislav Rončević[a,*], Damir Vukičević[b], Lucija Krce[a], Monica Benincasa[c], Ivica Aviani[a], Ana Maravić[d], Alessandro Tossi[c]

[a] Department of Physics, Faculty of Science, University of Split, Split, Croatia
[b] Department of Mathematics, Faculty of Science, University of Split, Split, Croatia
[c] Department of Life Sciences, University of Trieste, Trieste, Italy
[d] Department of Biology, Faculty of Science, University of Split, Split, Croatia

ARTICLE INFO

Keywords:
Anuran antimicrobial peptides
Peptide modifications
Selectivity index

ABSTRACT

Antimicrobial peptides (AMPs) are plausible candidates for the development of novel classes of antibiotics with a low tendency to elicit resistance. They often form lesions in the bacterial membrane making it hard for bacteria to develop permanent resistance. However, a potent antibacterial activity is often accompanied by excessive cytotoxicity towards host cells. Modifying known natural sequences, based on desirable biophysical properties, is expensive and time-consuming and often with limited success. 'Mutator' is a freely available web-based computational tool for suggesting residue variations that potentially increase a peptide's selectivity, based on the use of quantitative structure activity relationship (QSAR) criteria. Although proven to be successful, it has never been used to analyze multiple sequences simultaneously. Modifying the Mutator algorithm allowed screening of many sequences in the dedicated Database of Anuran Defense Peptides (DADP) and by implementing limited amino acid substitutions on appropriate candidates, propose 8 potentially selective AMPs called Dadapins. Two were chosen for testing, confirming the prediction and validating this approach. They were shown to efficiently inactivate bacteria by disrupting their membranes but to be non-toxic for host cells, as determined by flow cytometry and confirmed by atomic force microscopy (AFM).

1. Introduction

Bacterial resistance has become a widespread and increasingly serious problem in the last decades, equally for Gram-positive and Gram-negative bacterial species [1–3]. Due to a lack of new, effective antibiotics, colistin has become a 'drug of last resort' despite some toxic side effects [4], but in recent years its use has been limited by plasmidic dissemination of the *mcr-1* resistance gene [5]. Possible candidates for the development of new and effective antibiotics are AMPs - endogenous antibiotics present in all organisms with a direct cytotoxic activity against pathogens and often also showing useful immunomodulatory properties [6,7]. These peptides are known to be active against various bacterial species, and to use multimodal mechanisms that often involve interaction with bacterial membranes, making them alternative to most conventional antibiotics. They are therefore often active against multidrug resistant clinical pathogens [8] and it is hard for bacteria to develop permanent resistance against them. However, AMPs that exhibit potent antibacterial activity also often show an unacceptably high toxicity towards host cells [9]. It is not easy to identify, or to design peptides with desirable antimicrobial effects and an acceptable cytotoxicity, which is often quantified as a selectivity index ($SI = HC_{50} / MIC$), given by the ratio between the peptide concentration lysing 50% human red blood cells (HC_{50}) and the minimal inhibitory concentration (MIC) inhibiting bacteria growth.

With the development of next-generation sequencing techniques and abundance of publicly available genomic and transcriptomic data, high-throughput methods have recently been developed to identify putative novel AMPs [10,11] from genomic data. This may also allow

Abbreviations: DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; AFM, atomic force microscopy; AMPs, antimicrobial peptides; QSAR, quantitative structure activity relationship; DPPG, 1,2-dipalmitoyl-*sn*-phosphatidylglycerol; SPB, sodium phosphate buffer; SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid; TFE, trifluoroethanol

* Corresponding author.
E-mail addresses: troncevic@pmfst.hr (T. Rončević), vukicevi@pmfst.hr (D. Vukičević), lkrce@pmfst.hr (L. Krce), mbenincasa@units.it (M. Benincasa), iaviani@pmfst.hr (I. Aviani), amaravic@pmfst.hr (A. Maravić), atossi@units.it (A. Tossi).

Accepted 29 January 2019

analyses of multiple species simultaneously, and requires only small amounts of tissue [12]. However, even if effective in identifying potentially potent AMPs, these methods do not provide predictions regarding the balance of antimicrobial to cytotoxic effects.

Other types of approaches may include redesign of AMP sequences via statistical, template-based studies or to vary physico-chemical parameters that correlate with activity and selectivity [13]. They rely on known peptide sequences which are modified (often by single amino acid substitution) to improve antibacterial activity and/or toxicity towards host cells [14], and take into consideration relevant biophysical properties such as net charge, the hydrophobic/polar residue balance and resulting amphipathicity, and/or the tendency for self-aggregation, since all these features are closely linked to peptide activity and/or selectivity [15]. Other types of biophysical studies may include molecular dynamics simulations which can provide strong hypotheses and lead to novel peptide sequences, but are limited due to the constrained initial conditions and duration of simulation time [13]. Another type of promising approach is based on virtual screening studies where quantifiable properties of peptides (such as the abovementioned charge, amphipathicity and hydrophobicity) are used to construct appropriate molecular descriptors. Utilizing QSAR, the descriptors can then link biophysical properties, which act as input variables, with biological activity (potency, selectivity) as the output variable [13,16].

The 'Mutator' tool is a freely available web-based software (http://split4.pmfst.hr/mutator/) for improving peptide selectivity by using such quantitative SAR. It suggests appropriate mutations, limited to one or two amino-acid substitutions, to increase the selectivity index (SI) [17,18] which can be achieved either by decreasing peptide toxicity (i.e. increase the HC_{50} values), or by increasing antibacterial potency (i.e. decrease the MIC values), while not altering the toxicity. The software has been trained on helical AMPs of anuran origin, and therefore may provide best results if natural peptides of anuran origin are used as input. The dedicated Database of Anuran Defense Peptides (DADP) [19] is a valuable source for such sequences, and can be subjected to the Mutator tool in order to design new antimicrobials with high predicted SI. Instead of inputting peptides 'one by one', the algorithm was modified to automatically extract peptides from the DADP based on i) low MIC values against *Staphylococcus aureus*, ii) a specified peptide length and iii) appropriate SI values, before applying the limited mutations to potentially increase the SI. This resulted in eight new sequences, named Dadapin 1–8. All the peptides maintained appropriate features closely correlated with potent antibacterial activity, and two were selected for synthesis and verification. Based on the results of antimicrobial and haemolysis assay, both of these peptides proved to be moderately active against bacteria with very low toxicity towards red blood cells, confirming the usefulness of the described approach. This is despite the fact that both peptides still act via membrane disruption, as confirmed by flow cytometry and visualized by AFM.

2. Materials and methods

2.1. AMPs data set and Mutator software

The Mutator software implements limited residue variations on a given peptide sequence to increase the SI of the daughter sequence, as previously described in detail in Kamech et al. [17], based on the D-descriptor [18]. Briefly, its purpose is to increase SI without excessively disturbing regularities of the peptide primary and secondary structures. If the sequence is represented by residues A_1, $A_2 \ldots A_i \ldots A_n$, Mutator suggests that for residue A_i to be replaced by *Bi*, then this should be one of the five most frequent neighbors of A_{i-1} in a training set of peptides with good antimicrobial properties (20 peptides with highest SI among peptides in Table S1 of reference [18]). *Bi* should also be among the five most frequent successors of A_{i-4}, so that peptide amphipathicity should not be excessively decrease. Moreover, B_i must be either hydrophobic or polar in a manner coinciding with 4 predecessor and 4 successor amino-acids flanking it on a helical wheel projection. This algorithm takes into account the fact that many AMPs have helical conformations for at least part of their sequence, and attempts not to disrupt amphipathicity, as it is known to correlate with potent, membrane-directed activity. The global hydrophobicity of the peptide after mutation should also remain within a theoretically assumed optimal level (-1.5 to 0.5, normalized Eisenberg scale [20]).

The algorithm can be used as *Single* or *Double* implementations of *Mutator*, depending on whether one or two residues are altered, where the former is considered the stricter application. Furthermore, the stringency of the above requirements (see [18] for details) can be modified, leading to *Weak* or *Harsh* implementations of *Mutator* that can be combined with the *Single* or *Double* implementations. In the version used in this study, appropriate modifications were made to *Mutator* so that larger sets of peptide sequences, specifically derived from anuran defense peptides, could be inputted and analyzed successively, activating all implementation combinations.

DADP contains 2571 entries with 1923 non-identical bioactive sequences. Out of these, MIC data are available for 921 sequences [19]. Mutator was set so that only bioactive peptide sequences with reported MIC $\leq 3\,\mu M$ for *S. aureus* and length ≤ 25 amino acid residues were considered, resulting in a final data set of 62 peptides (see Table S1). Strict initial conditions were then used to further reduce the number of sequences subjected to the *Single* or *Double* and *Weak* or *Harsh Mutator* implementations (which refers to a greater or lesser stringency in applying the parameters for deciding the mutation) and outputted from it. An SI value threshold of ≥ 40 was set for the incoming parent peptide sequence, and only mutations resulting in an increase ≥ 10 of SI were accepted as output. Other requirements, as briefly described above, also had to be met. The minimal accepted SI value for selecting the final set of daughter peptides was then set to 80 (note that in the algorithm, SI ranges from 1 to 95). Verification of these daughter sequences suggested that these peptides would have biophysical properties that are strong indicators of antibacterial activity (e.g. secondary structure prediction, hydrophobicity, amphipathicity, etc.).

2.2. Peptide synthesis

Selected Dadapin peptides were obtained from GenicBio Limited (Shanghai, China), Dadapin-8 having a disulfide bridge and being C-terminally amidated, both at > 98% purity as confirmed by RP-HPLC and mass-spectrometry (see Fig. S1). Parent peptides, Odorranain-HP and Odorranain-B1 were also obtained (see Fig. S2). Chromatographic separation was achieved on a reversed-phase column (C18, 5 µm, 110 Å, 4.6 × 250 mm) using a 10–70% acetonitrile/0.1% TFA gradient in 25 min at a 1 ml/min flow rate. Peptide stock concentrations were determined by dissolving accurately weighed aliquots of peptide in doubly distilled water, and further verified by using the extinction coefficients at 214 nm, calculated as described by Kuipers and Gruppen [21].

2.3. Preparation of liposomes

LUVs (large unilamellar vesicles) were prepared by dissolving dry 1,2-dipalmitoyl-*sn*-phosphatidylglycerol (DPPG; anionic LUVs) (Avanti Polar Lipids, Alabaster, Alabama, USA) and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC; neutral LUVs) (Avanti Polar Lipids) in chloroform/methanol (2:1) solution. The solution was evaporated using a dry nitrogen stream and vacuum-dried for 24 h. The liposome cake was resuspended in 1 ml of sodium phosphate buffer (SPB, 10 mM, pH 7) to a concentration of 5 mM phospholipid and spun for 1 h at a temperature above the Tc (lipid critical temperature). The resulting multilamellar vesicle suspensions were disrupted by several freeze–thaw cycles prior to extrusion with a mini-extruder (Avanti Polar Lipids) through successive polycarbonate filters with 1 µm, 0.4 µm and 0.1 µm pores and resuspended to a final phospholipid concentration of 0.4 mM. Based on

the bilayer membrane surface area of a ~100 nm liposome, and area of a phospholipid head group (~0.7–1 nm^2) [22], the concentration of liposomes is about 5 nM.

2.4. Circular dichroism

CD spectra were obtained on a J-710 spectropolarimeter (Jasco, Tokyo, Japan). The spectra are accumulation of three scans measured in a) SPB solution, b) 50% TFE in SPB, c) the presence of sodium dodecyl sulfate micelles (10 mM SDS in SPB), d) the presence of anionic LUVs (DPPG) in SPB or e) the presence of neutral LUVs (DOPC) in SPB. The helix content was determined as $=[\theta]^{222}/[\theta]^{\alpha}$, where $[\theta]^{222}$ is the measured molar/residue ellipticity at 222 nm under any given condition and $[\theta]^{\alpha}$ is the molar ellipticity for a perfectly formed alpha helix of the same length, estimated as described by Chen et al. [23]. Secondary structure contribution was also determined with BeStSel (http://bestsel.elte.hu/index.php) as previously described by Micsonai et al. [24].

2.5. Antimicrobial activity

The *in vitro* testing was done on four standard Gram-negative laboratory strains including *E. coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 13883, *Acinetobacter baumannii* ATCC 19606 and *Pseudomonas aeruginosa* ATCC 27853 as well as *S. aureus* ATCC 29213 as a representative of Gram positives. Minimal inhibitory concentration was assessed using the serial two-fold microdilution method according to CLSI [25]. Bacteria were cultured in a fresh 20% Mueller Hinton broth (MHB) to the mid exponential phase, added to serial dilutions of Dadapin peptides to a final load of 5×10^5 CFU/ml in 100 µl per well, and incubated at 37 °C for 18 h. MIC was visually determined as the lowest concentration of the peptide showing no detectable bacterial growth and it was a consensus value of an experiment performed in triplicate.

For determination of minimal bactericidal concentration (MBC), 4 µL of bacterial suspensions were taken from the wells corresponding to MIC, 2 × MIC, and 4 × MIC and then plated on MH agar plates. Plates were incubated for 18 h at 37 °C to allow the viable colony counts and the MBC determined as the peptide concentration causing no visible growth.

2.6. Haemolysis assay

The cytotoxicity study of Dadapin peptides on human red blood cells observed the ethical principles of the Declaration of Helsinki and was determined by haemolysis assay. Peripheral blood samples were drawn from young and healthy male donor into vacutainers containing EDTA under aseptic conditions. The blood was washed twice with an ice cold PBS (10 mM phosphate buffer, pH 7.4), supernatant discarded, and the pellet resuspended in the equal amount of PBS. Then, the blood was diluted in PBS to a concentration of 1% (v/v) and 100 µl aliquots were added to an equal volume of peptide in PBS to a final concentration of 0.5% erythrocytes. Haemoglobin release was monitored at 450 nm using ELx808 Ultra Microplate Reader (BioTek, Inc., Winooski, VT, USA). Total lysis (100% haemolysis) was determined by the addition of 2% (v/v) Triton X-100, and the negative control value by the incubation of the red blood cells in PBS. The HC_{50} value was taken as the mean concentration of peptide producing 50% haemolysis. All evaluations were repeated as three separate experiments, using the same donor, carried out in triplicate, each time covering different concentration ranges.

2.7. Membrane integrity assay

The effect of Dadapin-1 and -8 on bacterial membrane integrity was studied by measuring the percentage of propidium iodide (PI) positive cells on exposure to the peptides, with a Cytomics FC 5000 flow cytometer (Beckman-Coulter, Inc., Fullerton, CA). Measurements were carried out on two reference strains, *S. aureus* ATCC 29213 and *K. pneumoniae* ATCC 13883 which were cultured in 20% MH broth to the mid-logarithmic phase. After incubation, PI was added to the bacterial suspension (1×10^6 CFU/ml) at a final concentration of 15 µM. Peptides were then added in different concentrations ranging from ¼ MIC to 2 × MIC just before the beginning of the analysis and the measurement taken at 15 min. Cells incubated in MH broth without peptides were used as negative control. Experiment was repeated at least three times and the data analysis was performed with the FCS Express3 software (De Novo Software, Los Angeles, CA, USA). Significance of differences between concentrations for each peptide was assessed by using InStat (GraphPad Software Inc., San Diego, CA, USA) and performed by an analysis of variance between groups (ANOVA) followed by the Student Newman-Keuls post-test. Values of $P < 0.05$ were considered statistically significant.

2.8. AFM imaging

The same reference strains which were used for membrane permeabilization studies were chosen for atomic force microscopy investigations. Over-night culture growth, next-day dilutions and treatments were carried out as reported in Rončević et al. [26]. Briefly, prepared cultures were treated with 2 × MIC concentrations of peptides for 1 h, briefly centrifuged and resuspended in 100 µl of the supernatant. The untreated bacterial cells were prepared in the same way but without the peptide treatment. Bacterial adhesion to glass slides was enabled with Cell-Tak solution (Corning, NY, USA) coating [27] as reported in [26]. AFM images were obtained under ambient conditions in contact mode using a Bruker Multimode 3 instrument (Digital Instruments, USA) with 0.12 N/m and 0.06 N/m silicon-nitride probes (Bruker AFM probes USA, DNPS-10). The image resolution was 512 pixels per line with scan rates between 2 and 3 Hz. Analysis of data was carried out with Gwyddion [28].

3. Results and discussion

3.1. Novel peptide sequences

Implementation of the chosen data set on the Mutator tool resulted in eight different peptide sequences named Dadapin 1–8, with significant point mutations and high predicted selectivity indices (see Table 1). Mutator has previously been shown to improve selectivity of selected anuran peptides, as high predicted SI correlated well with experimentally determined SI [14,17]. However, simultaneous automatic selection and mutation of multiple sequences had not been attempted before, and is novel, as most often methods for improving natural peptides are applied to single peptide sequence, chosen by expert verification, and the analogues rationally designed by manually substituting certain amino acids to obtain best possible biophysical properties [29–31]. Although they can be moderately successful in improving biological activity, these approaches are very time consuming and cannot be efficiently applied on the large amount of available sequences and activity data present in devoted AMP databases.

With the refinement of our Mutator algorithm we were successful in screening the entire DADP database using a defined set of parameters, resulting in a set of 62 peptides suitable for redesign. In the past, our main focus was to obtain peptides with acceptable SI when active against Gram-negative bacterial strains, since the initial algorithm was constructed based on a set of data for peptides with known MIC against *E. coli* (the most abundant set of data) and therefore should provide best output for similar strains [17]. However, peptide activity is frequently also reported for *S. aureus*, as a representative of Gram-positives (see below) and in this study we decided to build a dataset based on activity against this particular species. In any case, out of the subset of 62

Table 1
Dadapin peptides sequences and their physico-chemical characteristics.

Peptide[a]	Mutation[b]	Sequence[c]	Charge	[d]H	[e]μH[rel]	[f]SI[calc]
Dadapin-1	SM (V^8→ K)	GLLRASS**K**WGRKYYVDLAGCAKA	+5	−1.42 (−0.04)	0.42	88.7
Dadapin-2	DM (V^8→K, A^{21}→L)	GLLRASS**K**WGRKYYVDLAGC**L**KA	+5	−0.95 (−0.03)	0.46	94.8
Dadapin-3	SM (V^8→K)	GLFGKSS**K**WGRKYYVDLAGCAKA	+5	−1.46 (0)	0.39	86.8
Dadapin-4	DHM (F^3→S, R^{11}→V)	GL**S**GKSSVWG**V**KYYVDLAGCAKA	+3	−0.86 (0.21)	0.18	89.9
Dadapin-5	DM (G^4→K, W^9→Q)	GLF**K**KSSV**Q**GRKYYVDLAGCAKA	+5	−1.86 (−0.05)	0.32	94.6
Dadapin-6	DHM (A^7→K, R^{18}→L)	FLPKLF**K**KITKKNMAHI**L**	+6	0.31 (0.05)	0.38	94.9
Dadapin-7	DM (A^7→Q, R^{18}→L)	FLPKLF**Q**KITKKNMAHI**L**	+5	0.53 (0.09)	0.37	94.9
Dadapin-8	DM (L^3→K, K^9→V)	AA**K**KGCWT**V**SIPPKPCF-NH_2[g]	+4	−0.89 (0.14)	0.04	93.7

[a]The parent sequences for Dadapin-1, -2: Odorranain-HP (SI^{calc} = 73.3) (DADP ID: SP_A7YL71); Dadapin-3, -4, -5: Odorranain-W1 (SI^{calc} = 51.5) (DADP ID: SP_A6MBS8); Dadapin-6, -7: Andersonin-Y1 (SI^{calc} = 45.6) (DADP ID: SP_2843); Dadapin-8: Odorranain-B1 (SI^{calc} = 54.8) (DADP ID: SP_A6MBD6).
[b]SM = *Single Mutator*, DM = *Double Mutator*, DHM = *Double Harsh Mutator*.
[c]Measured MW (calculated MW): Dadapin-1 2513.96 (2513.92), Dadapin-8 1830.26 (1830.23); Odorranain-HP 2484.87 (2484.88); Odorranain-B1 2186.66 (2186.65). MW were calculated using PepCalc (https://pepcalc.com/).
[d]Calculated using the CCS consensus hydrophobicity scale [36], in brackets normalized Eisenberg scale data [20].
[e]Hydrophobic moment relative to a perfectly amphipathic helical peptide of 18 residues.
[f]Predicted selectivity index by using Mutator tool on a 1–95 scale.
[g]The parent sequence ends with an amidation signal (-GK, -GKR) [37].

peptides, appropriate modifications which would result in an appreciable predicted selectivity increase could be done on 4 peptides (Odorranain-HP, Odorranain-W1, Andersonin-Y1 and Odorranain-B1) resulting in a total of 8 new sequences. All of the output Dadapin sequences had suitable physico-chemical properties (e.g. adequate hydrophobicity, amphipathicity and charge, see Table 1) and a high predicted SI based on single or double amino acid substitutions.

Dadapin-1 and -8 were selected for synthesis and further experimental verification of their activity. Dadapin-1 was selected as the representative of the first sub-group of longer peptides (Dadapin 1–5) that have a similar charge and hydrophobicity (see Table 1). Given the fact those five sequences are very similar, selection was done based on the highest SI obtained with the stricter selection rule (single mutation) [17]. Out of the remaining three AMPs, Dadapin-8 was selected because its structure is the most different from the rest. It contains two Cys residues that likely engage in formation of a disulfide bridge, which should rather favor a β-hairpin-like conformation than a helical one, in line with the structure of the parent peptide, odoranain-B1 [32]. The presence of a disulfide bridge was a positive feature on inspection, as it has been reported to correlate with the activity for some frog derived peptides [33,34]. All the other peptides are linear and likely to adopt helical active structures to a greater or lesser extent, as is the case with many frog-derived peptides [35].

3.2. Peptide structure

The likely active structures of the two Dadapin peptides was determined by probing the effect of environment on conformation, using CD spectroscopy. Their spectra are consistent with mostly disordered structures in an aqueous environments (see Fig. 1), but a transition to partly ordered conformations is evident in the presence of membrane-like environments. Dadapin-1 shows a clear transition to a partly helical structure in the presence of TFE, although the helix content is estimated to be < 50%, and a strong contribution by disordered structure (> 70%) is also observed in the presence of anionic SDS micelles and DPPG LUVs, suggesting that it interacts with bacterial membranes only as a partially helical peptide. The effect is more pronounced for Dadapin-8, which shows a significant contribution from a β-hairpin-like conformation in the presence of anionic artificial membranes, (> 60%). Neither peptide shows a marked conformational change in the presence of neutral DOPC LUVs, suggesting that the peptides might not interact strongly with eukaryotic cell membranes, this and a reduced tendency to adopt a helical structure, could point to a reduced cytotoxicity [38].

3.3. Antimicrobial and haemolytic activity

The antibacterial activity of Dadapin peptides was assessed against reference ATCC strains of *S. aureus*, *E. coli*, *K. pneumoniae*, *P. aeruginosa* and *A. baumannii*. Both peptides were moderately active against Gram-negative bacteria (see Table 2) with Dadapin-1 being the more potent (MIC = 8 μM for most strains). On the other hand, the MIC value for *S. aureus* was 1 μM. The activity is significantly lower than that of the parent Odorranain-HP peptide, which has an MIC value of 8 μM against *S. aureus* and 32 μM against *E. coli*. It should be pointed out that these values are significantly higher than reported previously, although the test conditions were quite different [39,40].

Dadapin-8 was somewhat less potent, with MIC values of 16–32 μM, and in this case the best activity was against *K. pneumoniae* (MIC = 8 μM). MBC values were, in general, comparable to MIC suggesting the peptides are bactericidal rather than bacteriostatic (see Table 2). The lower potency of Dadapin-8 could be due to difference in structure compared to Dadapin-1, or its lower charge. Note that we used a C-terminally amidated peptide, given the presence of a GKR amidation sequence, leading to the decreased charge. We therefore tested the full sequence of the parent peptide Odorranain-B1, with charge of +6 (see Table 1) which turned out to be poorly active in our

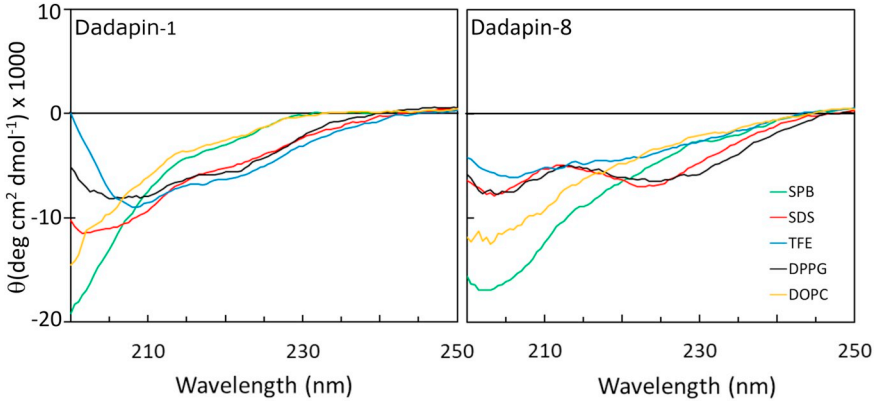

Fig. 1. CD spectra of Dadapin peptides under different conditions. Spectra are the accumulation of three scans carried out with 20 μM peptide in SPB, 10 mM SDS in SPB, 50% TFE, anionic LUVs in SPB (DPPG, 5 nM) and neutral LUVs in SPB (DOPC, 5 nM).

Table 2
Antimicrobial activity of Dadapin peptides and parent peptide sequences (Odorranains) measured as MIC (μM), along with HC_{50} values (μM) and selectivity index calculated based on experimental results (SI).

Bacterial strain	Dadapin-1			Odorranain-HP			Dadapin-8			Odorranain-B1		
	MIC	MBC	SI^{exp}	MIC	MBC	SI^{exp}	MIC	MBC	SI^{exp}	MIC	MBC	SI^{exp}
S. aureus (ATCC 29213)	1	1	670	8	8	63	16	32	> 60	> 64	> 64	NA
E. coli (ATCC 25922)	8–16	16–32	42–84	32	32	16	16–32	32	> 30	32	32	> 20
A. baumannii (ATCC 19606)	8	16	84	64	> 64	8	16	16	> 60	> 64	> 64	NA
K. pneumoniae (ATCC 13883)	8	32	84	16	16	32	8	8–16	> 120	> 64	> 64	NA
P. aeruginosa (ATCC 27853)	8	16	84	> 64	> 64	< 8	32	64	> 30	> 64	> 64	NA
Haemolytic activity (HC_{50})		670			500			> 1000			> 600	

NA not applicable.

hands. Again, this is significantly higher than the reported values of MIC for *S. aureus* and *E. coli* (~1 and 3 μM respectively) [32], likely due to different assay conditions. Thus, despite reducing the charge by amidation, we have managed to produce a modified peptide with appreciable activity.

Both Dadapins-1 and -8 were found to be quite non-toxic for red blood cells, with estimated HC_{50} values of ~700 μM and > 1 mM respectively (see Table 2 and Fig. 2). In fact, it was not possible to reach the HC_{50} value experimentally, but just extrapolate it. This may be more reliably estimated in the case of Dadapin-1, due to the linear correlation between concentration and haemolysis (see Fig. 2), while for Dadapin-8, little cell lysis was observed up to 800 μM (< 15%), with a sudden increase to about 50% at 1000 μM. In any case, comparing the haemolysis with that of the parent peptides Odorranain-HP and B1, the modified peptides have an equally low, if not lower toxicity (500 and > 600 μM respectively, Fig. 3 and Table 2). It is worth noting, for Odorranain-HP, the center of the upper cluster was conservatively used for the determination of HC_{50} (see Fig. 3), which could have been determined as 350 or 400 μM (separator of two clusters).

Both peptides therefore showed moderate activity against bacteria but quite low cytotoxicity, resulting in promising selectivity indices especially considering the effect of Dadapin-1 on *S. aureus* (SI = 670) and Dadapin-8 on *K. pneumoniae* (SI > 120). It is worth noting however, that the peptides' antibacterial activity was found to be medium-sensitive and decreased several-fold in full MH medium (data not shown). This emphasizes the difficulty of designing adequate peptide/s for possible biomedical applications even when introducing subtle changes in the structure of naturally honed peptide.

3.4. Bacterial membrane permeability

Flow cytometry was used to monitor the effect of Dadapins on the integrity of the cytoplasmic bacterial membrane, since PI incorporates into nucleic acids and becomes fluorescent only in bacterial cells with

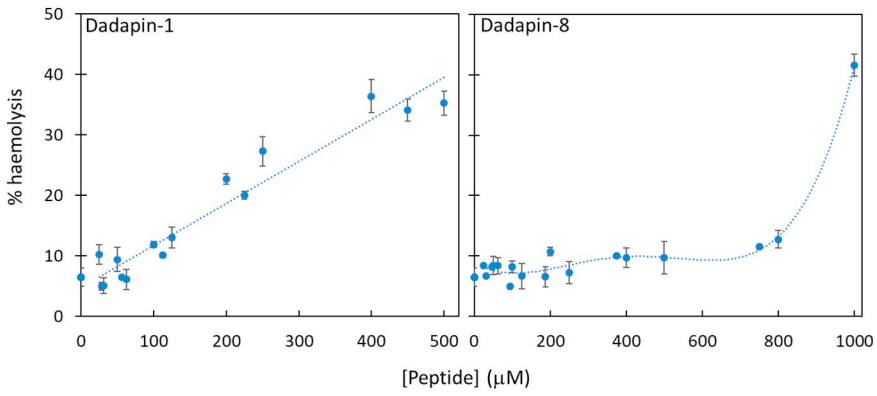

Fig. 2. Haemolytic activity of Dadapin peptides. Human erythrocytes were treated with peptides at increasing concentrations in PBS. Haemolysis was determined by measuring the absorbance of the supernatant at 450 nm after 1 h incubation of erythrocytes with each peptide at 37 °C and compared to the values achieved by treatment with 2% Triton X-100. Negative control point (peptide concentration = 0 μM) was determined by the incubation of the red blood cells in PBS. Each data point represents the mean value of one experiment performed in triplicate ± SD.

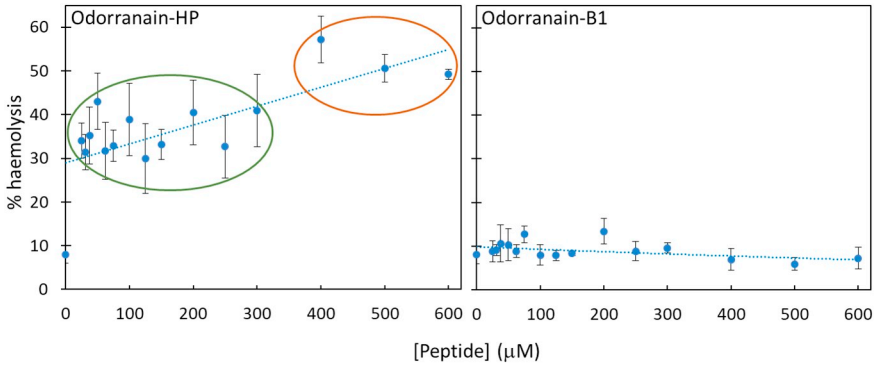

Fig. 3. Haemolytic activity of Odorranain peptides. Human erythrocytes were treated with peptides at increasing concentrations in PBS. Haemolysis was determined by measuring the absorbance of the supernatant at 450 nm after 1 h incubation of erythrocytes with each peptide at 37 °C and compared to the values achieved by treatment with 2% Triton X-100. Negative control point (peptide concentration = 0 μM) was determined by the incubation of the red blood cells in PBS. Each data point represents the mean value of one experiment performed in triplicate ± SD. Lower cluster indicates concentrations not reaching 50% haemolysis and right cluster indicates points with concentrations reaching HC_{50}.

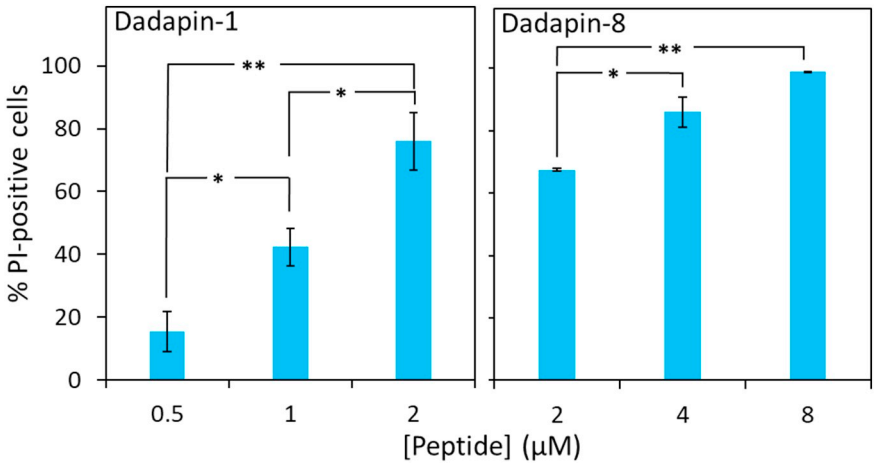

Fig. 4. Evaluation of the effect of Dadapin peptides on bacterial membrane integrity. Dadapin-1 was incubated with *S. aureus* (1×10^6 CFU/ml) and Dadapin-8 with *K. pneumoniae* (1×10^6 CFU/ml) for 15 min at concentrations equal to ¼ MIC, ½ MIC, MIC or 2 × MIC. Data represent % PI+ treated cells subtracted of the % PI+ untreated control cells (< 2%). They are expressed as the mean of % PI positive cells ± SEM of at least three independent experiments (*$P < 0.05$, **$P < 0.005$, Student Newman-Keuls post-test).

membrane damage. The experiment was carried out with either *S. aureus* ATCC 29213 and *K. pneumoniae* ATCC 13883, as Dadapin-1 and -8 were most active against these particular strains respectively (see Table 1).Treatment of *S. aureus* with Dadapin-1 ATCC 29213 caused > 70% permeabilization at 2 μM (2 × MIC) after 15 min and appreciable permeabilization (> 40% PI+ cells) at MIC values (1 μM). Further decrease in peptide concentration to ½ MIC (0.5 μM) resulted in ~20% PI+ cells, indicating that the peptide is capable of disrupting the membrane or altering its barrier function even at sub-MIC concentrations (see Fig. 4). Treatment of *K. pneumoniae* ATCC 13883 with Dadapin-8 caused a more pronounced effect, with > 60% PI+ cells already after incubation with the peptide at a concentration corresponding to ¼ MIC (2 μM). At ½ MIC and MIC concentrations the % of PI+ cells increased to ~90% and ~100%, respectively, indicating a strong membranolytic effect which is typical for AMPs with β-hairpin fold [41]. Prolonging the incubation time for both peptides and both strains did not result in the increase of % of PI+ cells.

3.5. AFM images

Membrane damage on *S. aureus* ATCC 29213 and *K. pneumoniae* ATCC 13883 was visualized with AFM after cell treatment with peptide concentrations corresponding to 2 × MIC. The surface of untreated *S. aureus* cells was relatively smooth, and the cell wall seems to be preserved. The polysaccharide capsule of untreated *K. pneumoniae* was also well preserved, with visible pili (see Fig. 5a and c). Treated *S. aureus* cells, on the other hand, seemed blistered and the cell wall not as smooth. Furthermore, some of the cells appeared to be ruptured and surrounded with possible intracellular matter (see Fig. 5b). Similarly, *K. pneumoniae* cells treated with Dadapin-8 cells were evidently altered and surface roughness quite evidently changed (see Fig. 5d). Based on both membrane permeabilization and AFM studies, carried out at comparable peptide concentrations, it can be concluded that both peptides act by a membrane disruption, although the effect seems to be somewhat less pronounced for Dadapin-1.

4. Conclusions

Applying the Mutator algorithm to a large set of peptides present in a dedicated anuran AMP sequence/activity database allowed preparation of a novel group of such peptides; the Dadapins. From this, two structurally very different peptides were selected for synthesis and testing, and showed promising selectivity indices, associated in the case of Dadapin-1 with a potent activity against *S. aureus*. Despite their structural differences, both peptides proved to be membrane active disrupting the bacterial membrane even at sub-MIC concentrations. The method did not limit selection to linear, helical peptides, but resulted also in a bridged, β-sheet AMP with appreciable activity against *K. pneumoniae*. Taken together, our findings suggest the approach presented in this paper can be further exploited to develop novel peptides with limited host cell toxicity and active against various bacterial strains. In particular Dadapin-1 shows a good activity with negligible toxicity on erythrocytes and bears further investigation. Evaluation of cytotoxic effects on mammalian cells other than erythrocytes can be a subject of future additional *in vitro* testing as well as NMR structural studies and dye release assays on liposomes in order to get a deeper

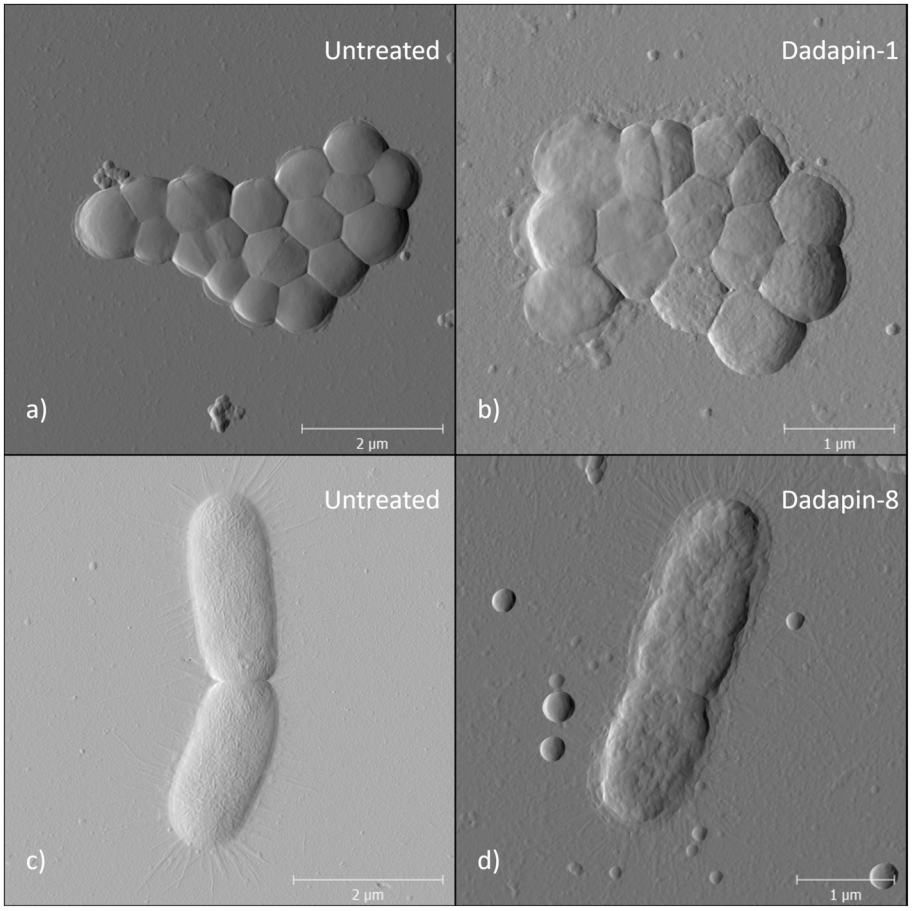

Fig. 5. AFM deflection images of untreated *S. aureus* [panel a)] and *K. pneumoniae* [panel c)] cells and in the presence of Dadapin-1 [panel b)] and Dadapin-8 [panel d)], respectively. Bacteria were exposed to the Dadapins at concentrations equivalent to 2 × MIC.

understanding of mode of action, as well as possibly suggesting modifications to improve salt-sensitivity.

Conflict of interest

The authors declare no conflict of interest.

Transparency document

The Transparency document associated with this article can be found, in online version.

CRediT authorship contribution statement

Tomislav Rončević: Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft, Writing - review & editing, Visualization. **Damir Vukičević:** Conceptualization, Methodology, Software, Formal analysis, Investigation, Resources, Data curation, Funding acquisition. **Lucija Krce:** Methodology, Investigation, Formal analysis, Visualization. **Monica Benincasa:** Investigation. **Ivica Aviani:** Formal analysis, Funding acquisition. **Ana Maravić:** Investigation. **Alessandro Tossi:** Methodology, Formal analysis, Writing - original draft, Writing - review & editing, Supervision.

Acknowledgments

The authors acknowledge funding from Croatian Science Foundation [grant number 8481].

References

[1] I. Roca, M. Akova, F. Baquero, J. Carlet, M. Cavaleri, S. Coenen, J. Cohen, D. Findlay, I. Gyssens, O.E. Heure, G. Kahlmeter, H. Kruse, R. Laxminarayan, E. Liébana, L. López-Cerero, A. MacGowan, M. Martins, J. Rodríguez-Baño, J.-M. Rolain, C. Segovia, B. Sigauque, E. Tacconelli, E. Wellington, J. Vila, The global threat of antimicrobial resistance: science for intervention, New Microbes New Infect. 6 (2015) 22–29, https://doi.org/10.1016/j.nmni.2015.02.007.

[2] L.L. Maragakis, T.M. Perl, Acinetobacter baumannii: epidemiology, antimicrobial resistance, and treatment options, Clin. Infect. Dis. 46 (2008) 1254–1263, https://doi.org/10.1086/529198.

[3] F.D. Lowy, Antimicrobial resistance: the example of Staphylococcus aureus, J. Clin. Invest. 111 (2003) 1265–1273, https://doi.org/10.1172/JCI18535.

[4] A. Ordooei Javan, S. Shokouhi, Z. Sahraei, A review on colistin nephrotoxicity, Eur. J. Clin. Pharmacol. 71 (2015) 801–810, https://doi.org/10.1007/s00228-015-1865-4.

[5] Y.-Y. Liu, Y. Wang, T.R. Walsh, L.-X. Yi, R. Zhang, J. Spencer, Y. Doi, G. Tian, B. Dong, X. Huang, L.-F. Yu, D. Gu, H. Ren, X. Chen, L. Lv, D. He, H. Zhou, Z. Liang, J.-H. Liu, J. Shen, Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study, Lancet Infect. Dis. 16 (2016) 161–168, https://doi.org/10.1016/S1473-3099(15)00424-7.

[6] A. Nijnik, R. Hancock, Host defence peptides: antimicrobial and immunomodulatory activity and potential applications for tackling antibiotic-resistant infections, Emerg. Health Threats J. 2 (2009), https://doi.org/10.3134/ehtj.09.001.

[7] A. Cederlund, G.H. Gudmundsson, B. Agerberth, Antimicrobial peptides important in innate immunity: antimicrobial peptides important in innate immunity, FEBS J. 278 (2011) 3942–3951, https://doi.org/10.1111/j.1742-4658.2011.08302.x.

[8] S.-C. Park, Y. Park, K.-S. Hahm, The role of antimicrobial peptides in preventing multidrug-resistant bacterial infections and biofilm formation, Int. J. Mol. Sci. 12

(2011) 5971–5992, https://doi.org/10.3390/ijms12095971.
[9] B.K. Pandey, A. Ahmad, N. Asthana, S. Azmi, R.M. Srivastava, S. Srivastava, R. Verma, A.L. Vishwakarma, J.K. Ghosh, Cell-selective lysis by novel analogues of melittin against human red blood cells and Escherichia coli, Biochemistry (Mosc) 49 (2010) 7920–7929, https://doi.org/10.1021/bi100729m.
[10] Y. Yi, X. You, C. Bian, S. Chen, Z. Lv, L. Qiu, Q. Shi, High-throughput identification of antimicrobial peptides from amphibious mudskippers, Mar. Drugs 15 (2017) 364, https://doi.org/10.3390/md15110364.
[11] B. Dong, Y. Yi, L. Liang, Q. Shi, High throughput identification of antimicrobial peptides from fish gastrointestinal microbiota, Toxins 9 (2017), https://doi.org/10.3390/toxins9090266.
[12] T. Rončević, M. Gerdol, F. Spazzali, F. Florian, S. Mekinić, A. Tossi, A. Pallavicini, Parallel identification of novel antimicrobial peptide sequences from multiple anuran species by targeted DNA sequencing, BMC Genomics 19 (2018), https://doi.org/10.1186/s12864-018-5225-5.
[13] C.D. Fjell, J.A. Hiss, R.E.W. Hancock, G. Schneider, Designing antimicrobial peptides: form follows function, Nat. Rev. Drug Discov. (2011), https://doi.org/10.1038/nrd3591.
[14] T. Rončević, G. Gajski, N. Ilić, I. Goić-Barišić, M. Tonkić, L. Zoranić, J. Simunić, M. Benincasa, M. Mijaković, A. Tossi, D. Juretić, PGLa-H tandem-repeat peptides active against multidrug resistant clinical bacterial isolates, Biochim. Biophys. Acta Biomembr. 1859 (2017) 228–237, https://doi.org/10.1016/j.bbamem.2016.11.011.
[15] Z. Jiang, A.I. Vasil, J.D. Hale, R.E.W. Hancock, M.L. Vasil, R.S. Hodges, Effects of net charge and the number of positively charged residues on the biological activity of amphipathic α-helical cationic antimicrobial peptides, Biopolymers 90 (2008) 369–383, https://doi.org/10.1002/bip.20911.
[16] Hå. Jenssen, C.D. Fjell, A. Cherkasov, R.E.W. Hancock, QSAR modeling and computer-aided design of antimicrobial peptides, J. Pept. Sci. 14 (2008) 110–114, https://doi.org/10.1002/psc.908.
[17] N. Kamech, D. Vukičević, A. Ladram, C. Piesse, J. Vasseur, V. Bojović, J. Simunić, D. Juretić, Improving the selectivity of antimicrobial peptides from anuran skin, J. Chem. Inf. Model. 52 (2012) 3341–3351, https://doi.org/10.1021/ci300328y.
[18] D. Juretić, D. Vukičević, N. Ilić, N. Antcheva, A. Tossi, Computational design of highly selective antimicrobial peptides, J. Chem. Inf. Model. 49 (2009) 2873–2882, https://doi.org/10.1021/ci900327a.
[19] M. Novkovic, J. Simunic, V. Bojovic, A. Tossi, D. Juretic, DADP: the database of anuran defense peptides, Bioinformatics 28 (2012) 1406–1407, https://doi.org/10.1093/bioinformatics/bts141.
[20] S. Kawashima, P. Pokarowski, M. Pokarowska, A. Kolinski, T. Katayama, M. Kanehisa, AAindex: amino acid index database, progress report 2008, Nucleic Acids Res. 36 (2008) D202–D205, https://doi.org/10.1093/nar/gkm998.
[21] B.J.H. Kuipers, H. Gruppen, Prediction of molar extinction coefficients of proteins and peptides using UV absorption of the constituent amino acids at 214 nm to enable quantitative reverse phase high-performance liquid chromatography–mass spectrometry analysis, J. Agric. Food Chem. 55 (2007) 5445–5451, https://doi.org/10.1021/jf070337l.
[22] A. Tossi, L. Sandri, A. Giangaspero, Amphipathic, α-helical antimicrobial peptides, Pept. Sci. 55 (2000) 4–30.
[23] Y.-H. Chen, J.T. Yang, K.H. Chau, Determination of the helix and β form of proteins in aqueous solution by circular dichroism, Biochemistry (Mosc) 13 (1974) 3350–3359.
[24] A. Micsonai, F. Wien, L. Kernya, Y.-H. Lee, Y. Goto, M. Réfrégiers, J. Kardos, Accurate secondary structure prediction and fold recognition for circular dichroism spectroscopy, Proc. Natl. Acad. Sci. U. S. A. 112 (2015) E3095–E3103, https://doi.org/10.1073/pnas.1500851112.
[25] Clinical and Laboratory Standards Institute, Performance Standards for Antimicrobial Susceptibility Testing, (2017).
[26] T. Rončević, D. Vukičević, N. Ilić, L. Krce, G. Gajski, M. Tonkić, I. Goić-Barišić, L. Zoranić, Y. Sonavane, M. Benincasa, D. Juretić, A. Maravić, A. Tossi, Antibacterial activity affected by the conformational flexibility in glycine–lysine based α-helical antimicrobial peptides, J. Med. Chem. 61 (2018) 2924–2936, https://doi.org/10.1021/acs.jmedchem.7b01831.
[27] R. Louise Meyer, X. Zhou, L. Tang, A. Arpanaei, P. Kingshott, F. Besenbacher, Immobilisation of living bacteria for AFM imaging under physiological conditions, Ultramicroscopy 110 (2010) 1349–1357, https://doi.org/10.1016/j.ultramic.2010.06.010.
[28] D. Nečas, P. Klapetek, Gwyddion: an open-source software for SPM data analysis, Open Phys. 10 (2012), https://doi.org/10.2478/s11534-011-0096-2.
[29] Z. Jiang, A.I. Vasil, L. Gera, M.L. Vasil, R.S. Hodges, Rational design of α-helical antimicrobial peptides to target gram-negative pathogens, Acinetobacter baumannii and Pseudomonas aeruginosa: utilization of charge, 'specificity determinants,' total hydrophobicity, hydrophobe type and location as design para: antimicrobial peptides to target Gram-negative pathogens, Chem. Biol. Drug Des. 77 (2011) 225–240, https://doi.org/10.1111/j.1747-0285.2011.01086.x.
[30] B. Deslouches, J.D. Steckbeck, J.K. Craigo, Y. Doi, T.A. Mietzner, R.C. Montelaro, Rational design of engineered cationic antimicrobial peptides consisting exclusively of arginine and tryptophan, and their activity against multidrug-resistant pathogens, Antimicrob. Agents Chemother. 57 (2013) 2511–2521, https://doi.org/10.1128/AAC.02218-12.
[31] I. Zelezetsky, U. Pag, H.-G. Sahl, A. Tossi, Tuning the biological properties of amphipathic α-helical antimicrobial peptides: rational use of minimal amino acid substitutions, Peptides 26 (2005) 2368–2376, https://doi.org/10.1016/j.peptides.2005.05.002.
[32] J. Li, X. Xu, C. Xu, W. Zhou, K. Zhang, H. Yu, Y. Zhang, Y. Zheng, H.H. Rees, R. Lai, D. Yang, J. Wu, Anti-infection peptidomics of amphibian skin, 6 (2007) 882–894.
[33] S.S.L. Harwig, A. Waring, H.J. Yang, Y. Cho, L. Tan, R.I. Lehrer, Intramolecular disulfide bonds enhance the antimicrobial and lytic activities of protegrins at physiological sodium chloride concentrations, Eur. J. Biochem. 240 (1996) 352–357, https://doi.org/10.1111/j.1432-1033.1996.0352h.x.
[34] N. Sitaram, K.P. Sai, S. Singh, K. Sankaran, R. Nagaraj, Structure-function relationship studies on the frog skin antimicrobial peptide tigerinin 1: design of analogs with improved activity and their action on clinical bacterial isolates, Antimicrob. Agents Chemother. 46 (2002) 2279–2283, https://doi.org/10.1128/AAC.46.7.2279-2283.2002.
[35] M.-A. Sani, F. Separovic, How membrane-active peptides get into lipid membranes, Acc. Chem. Res. 49 (2016) 1130–1138, https://doi.org/10.1021/acs.accounts.6b00074.
[36] A. Tossi, L. Sandri, A. Giangaspero, New consensus hydrophobicity scale extended to non-proteinogenic amino acids, Peptides 27 (2002) 416.
[37] R.G. Solstad, C. Li, J. Isaksson, J. Johansen, J. Svenson, K. Stensvåg, T. Haug, Novel antimicrobial peptides EeCentrocins 1, 2 and EeStrongylocin 2 from the edible sea urchin Echinus esculentus have 6-Br-Trp post-translational modifications, PLoS One 11 (2016) e0151820, , https://doi.org/10.1371/journal.pone.0151820.
[38] I. Zelezetsky, A. Tossi, Alpha-helical antimicrobial peptides—using a sequence template to guide structure–activity relationship studies, Biochim. Biophys. Acta Biomembr. 1758 (2006) 1436–1449, https://doi.org/10.1016/j.bbamem.2006.03.021.
[39] L. Chen, Y. Li, J. Li, X. Xu, R. Lai, Q. Zou, An antimicrobial peptide with antimicrobial activity against Helicobacter pylori, Peptides 28 (2007) 1527–1531, https://doi.org/10.1016/j.peptides.2007.07.007.
[40] X. Xu, J. Li, Y. Han, H. Yang, J. Liang, Q. Lu, R. Lai, Two antimicrobial peptides from skin secretions of Rana grahami, Toxicon 47 (2006) 459–464, https://doi.org/10.1016/j.toxicon.2006.01.002.
[41] R. Mani, A.J. Waring, R.I. Lehrer, M. Hong, Membrane-disruptive abilities of β-hairpin antimicrobial peptides correlate with conformation and activity: a 31P and 1H NMR study, Biochim. Biophys. Acta Biomembr. 1716 (2005) 11–18, https://doi.org/10.1016/j.bbamem.2005.08.008.