Identification, redesign and characterization of anuran antimicrobial peptides

Rončević, Tomislav

UNIVERSITY OF SPLIT

FACULTY OF SCIENCE

Postgraduate University Study of Biophysics

Doctoral thesis

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From targeted DNA sequencing and database analyses to peptide antibiotics

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IDENTIFICATION, REDESIGN AND CHARACTERIZATION OF ANURAN ANTIMICROBIAL PEPTIDES (*From targeted DNA sequencing and database analyses to peptide antibiotics*)

Tomislav Rončević completed his doctoral thesis under the supervision of Prof. Jasna Puizina and Assoc. Prof. Alessandro Tossi as a necessary obligation to attain a Ph.D. title.

Achieved academic title: Ph.D. in Science, field of Biology

Composition of the Expert Panel for the Assessment and Defence of the Doctoral Thesis:

1. Assist. Prof. Ana Maravić _____

2. Assoc. Prof. Karl Lohner _____

3. Assoc. Prof. Maria Luisa Mangoni _____

4. Assist. Prof. Larisa Zoranić _____
(substitute member)

We confirm that the Ph.D. thesis has been defended on the 25th April 2019.

Coordinator of the Study: Prof. Mile Dželalija _____

DEAN:

_____
Prof. Nikola Koceić-Bilan

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BASIC DOCUMENTATION CARD

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Faculty of Science Doctoral thesis

IDENTIFICATION, REDESIGN AND CHARACTERIZATION OF ANURAN ANTIMICROBIAL PEPTIDES

(*From targeted DNA sequencing and database analyses to peptide antibiotics*)

Tomislav Rončević

Thesis performed at:
- Faculty of Science, University of Split, Croatia
- Department of Life Sciences, University of Trieste, Italy

Abstract

Antimicrobial peptides (AMPs) are structurally diverse molecules naturally produced by most organisms, with direct antimicrobial activity against pathogens and often showing other immune-related properties. Anurans are a particularly rich source of these peptides, with ~2000 different sequences reported in Database of Anuran Defense Peptides (DADP). A novel approach for simultaneous multi-species peptide identification has been developed by exploiting the highly conserved signal peptide region to design family specific forward degenerate primers, using RNAseq data available in the Sequence Read Archive (SRA) database, and using them to selectively amplify AMP-coding transcripts from small amounts of frog skin tissue. Additionally, novel peptides have been redesigned with the help of molecular descriptors using the Mutator software that links biophysical properties of the peptides with their biological activity. This software has been trained on helical AMPs of anuran origin and may provide best results if natural peptides of anuran origin are used as input. In both cases, a number of the identified/designed peptides have been tested for antibacterial activity, toxicity and mode of action. Some of these proved to be potent antimicrobials with limited toxicity towards host cells.

(86 pages, 19 figures, 7 tables, 198 references, 2 appendices, original in English)
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Keywords: antimicrobial peptides, anura, peptide aggregation, membrane active peptides, peptide redesign, peptide selectivity, signal peptide region, targeted DNA sequencing

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Assoc. Prof. Alessandro Tossi

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IDENTIFIKACIJA, REDIZAJN I KARAKTERIZACIJA ANTIMIKROBNIH PEPTIDA IZ ŽABA (ANURA)

(*Od ciljanog sekvenciranja DNK i analiza baza podataka prema peptidnim antibioticima*)

Tomislav Rončević

Rad je izrađen u:

- Prirodoslovno-matematičkom fakultetu, Sveučilište u Splitu, Hrvatska
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Sažetak

Antimikrobni peptidi (AMP) su strukturalno različite molekule prirodno sintetizirane u većini organizmima, s direktnom antimikrobnom aktivnosti, dok često pokazuju i druga imunološki povezana svojstva. Vodozemci iz reda Anura su naročito bogat izvor takvih peptida s ~2000 različite sekvence pohranjene u 'Database of Anuran Defense Peptides' (DADP). Razvijen je novi pristup za istovremenu identifikaciju peptida iz više različitih vrsta oslanjajući se na izrazito očuvane signalne sekvence za dizajn specifičnih početnica i podatke RNAseq dostupne u 'Sequence Read Archive' (SRA), te ih koristeći kako bi se selektivno umnožili transkripti koji kodiraju AMP iz malih količina tkiva kože žaba. Također, novi peptidi su redizajnirani uz pomoć molekularnih deskriptora koristeći računalni program Mutator koji povezuje biofizikalna svojstva peptida s njihovom biološkom aktivnošću. Taj alat razvijen je koristeći set helikalnih AMP-a žabljeg podrijetla, te bi trebao dati najbolje rezultate ako se unose upravo takvi prirodni peptidi. U oba slučaja, određeni broj identificiranih/dizajniranih peptida je testiran naspram antibakterijske aktivnosti, toksičnosti i mehanizma djelovanja. Neki od tih peptida su se pokazali kao dobri antimikrobni kandidati s neznatnom toksičnošću naspram stanica domaćina.

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Ključne riječi: agregacija peptida, antimikrobni peptidi, anura, ciljano sekvenciranje DNK, membranski aktivni peptidi, redizajn peptida, regija signalnog peptida, selektivnost peptida

Mentori: Prof. dr. sc. Jasna Puizina
Izv. prof. dr. sc. Alessandro Tossi

Ocjenjivači: 1. Doc. dr. sc. Ana Maravić
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3. Izv. prof. dr. sc. Maria Luisa Mangoni

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The following publications constitute the main part of the thesis:

[1] 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). doi:10.1186/s12864-018-5225-5.

[2] T. Rončević, L. Krce, M. Gerdol, S. Pacor, M. Benincasa, F. Guida, I. Aviani, V. Čikeš-Čulić, A. Pallavicini, A. Maravić, A. Tossi, Membrane-active antimicrobial peptide identified in Rana arvalis by targeted DNA sequencing, Biochim. Biophys. Acta BBA - Biomembr. 1861 (2019) 651–659. doi:10.1016/j.bbamem.2018.12.014.

[3] T. Rončević, D. Vukičević, L. Krce, M. Benincasa, I. Aviani, A. Maravić, A. Tossi, Selection and redesign for high selectivity of membrane-active antimicrobial peptides from a dedicated sequence/function database, Biochim. Biophys. Acta BBA - Biomembr. 1861 (2019) 827–834. doi:10.1016/j.bbamem.2019.01.017.

I INTRODUCTION

1 Antibiotics and antimicrobial resistance – history is important

Despite some misconceptions, exposure to antibiotics is not confined to the modern antibiotic era starting from the early 20th century. It is now known that ancient civilizations had been using antibiotics to treat bacterial infections, and as such, topical applications of moldy bread are well documented from ancient Egypt, China, Greece and Rome [1]. Even some modern day antibiotics, the tetracyclines, have been found in traces in skeletal remains from ancient Sudanese Nubia (dating from 350 – 550 A.D.) and from the Roman period in Egypt [2–4]. In both cases, it is presumed that the antibiotic was digested through grains contaminated with *Streptomycetes* [3,4] and most likely acted with a preventative role rather than as a "systemic" treatment. Similarly, some remedies used in traditional Chinese medicine have been the source of potent antimicrobials for millennia, with artemisinin or "qinghaosu", a potent anti-malarial drug, being one of the best known examples [5].

The modern antibiotic era begins with work carried out by Paul Erlich and his concept of the "magic bullet" at the beginning of the 20th century. Together with the chemist Alfred Bertheim and bacteriologist Sahachiro Hata, he discovered the arsenic dye arsphenamine, later called Salvarsan or 606 (it was the 606th compound tested) which proved to be potent against syphilis [2,6]. However, in the strict sense of the word this was antimicrobial chemotherapy rather than antibiotic therapy. First widely available antibiotic, introduced in 1935 by Gerhard Domagk, was sulfonamidochrysoidine or Prontosil which had antibacterial activity in a number of infectious diseases. Shortly after, it was determined that Prontosil is in fact a precursor to *p*-aminophenylsulfonamide, already discovered in 1908 and thus not patentable, but which led to easily modified derivatives and the era of sulfonamide antibiotics [7]. Penicillin, one of the best known antibiotics, was discovered in 1928 by Alexander Fleming, although mass production started more than 15 years later during World War II, following synthesis and purification work carried out by Howard Florey and Ernest Chain [2,8]. In 1944, Selman Waksman, considered "the father of antibiotics" who first coined the term, discovered an aminoglycoside antibiotic from *Streptomyces griseus* and named it streptomycin [9,10]. This marked the beginning of the golden age of antibiotics, which led to the discovery of more than 20 different groups in the following decades [11], divided into 8 different classes: *β-lactams* (which include subclasses of penicillins, cephalosporins, monobactams and carbapenems), *macrolides*, *tetracyclines*, *quinolones*, *aminoglycosides*, *sulfonamides*, *glycopeptides* and *oxazolidinones* [12] (see **Figure 1**).

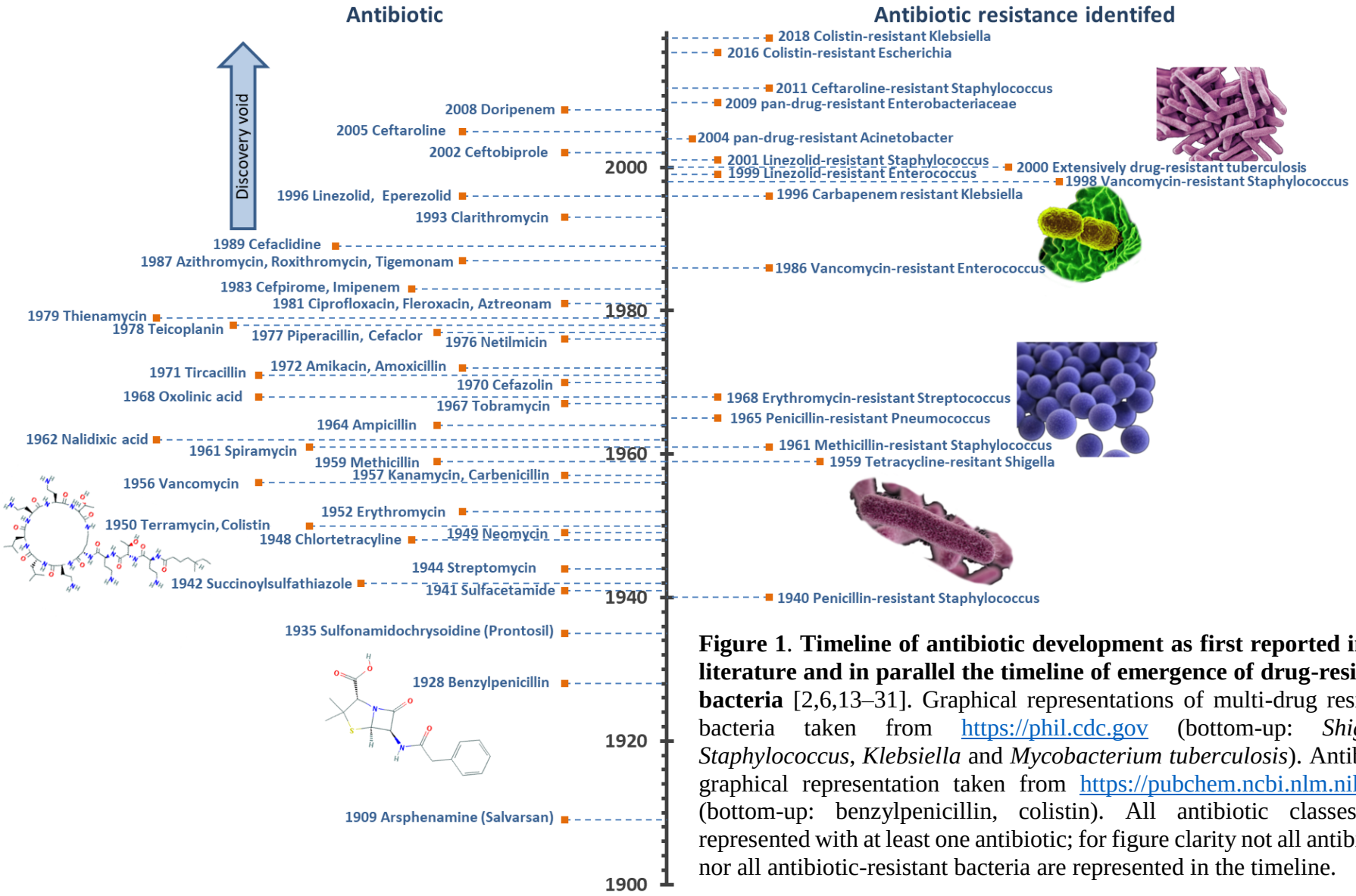

Figure 1. **Timeline of antibiotic development as first reported in the literature and in parallel the timeline of emergence of drug-resistant bacteria** [2,6,13–31]. Graphical representations of multi-drug resistant bacteria taken from https://phil.cdc.gov (bottom-up: *Shigella*, *Staphylococcus*, *Klebsiella* and *Mycobacterium tuberculosis*). Antibiotic graphical representation taken from https://pubchem.ncbi.nlm.nih.gov (bottom-up: benzylpenicillin, colistin). All antibiotic classes are represented with at least one antibiotic; for figure clarity not all antibiotics nor all antibiotic-resistant bacteria are represented in the timeline.

Without a doubt, antibiotics have changed the course of medicine and saved millions of lives worldwide since they were first introduced. Infectious diseases, and their catastrophic side effects, which could not previously be stopped, were now easily controlled. However, the application of the first antibiotics was followed not much later by the emergence of antibiotic resistance. Antibiotic resistance to penicillin in *Staphylococcus* had already emerged by 1940, few years before mass production had even begun, while resistance towards methicillin was first reported only 2 years after that drug has been introduced (see **Figure 1**). Consequently, additional novel antibiotics were discovered or developed and put into clinical use. Vancomycin, an antibiotic in the glycopeptide class, was first reported in the early 1950s and put to use in 1972 [27]. At the time, it was believed to be very unlikely for vancomycin resistance to occur in clinical practice. However, by 1986, vancomycin-resistant Enterococcus was isolated and approximately 10 years later, first reports of vancomycin-resistant *Staphylococcus* had emerged (see **Figure 1**). Nowadays, multidrug-resistant bacteria have become a major concern, especially the pan-drug resistant or extensively-drug resistant strains (see **Figure 1**) such as *Mycobacterium tuberculosis* resistant to fluoroquinolones and to any of the second-line injectable drugs (capreomycin, kanamycin, or amikacin) [28]. It is conservatively estimated that in the US and Europe 2.5 million people are affected by such infections each year, and 50,000 die as a result of the infection [28].

Another major concern is the lack of novel classes of antibiotics entering the pipeline over the last 30 years (see **Figure** 1). Some promising compounds are at various stages of clinical development [32], but few of these, if any, can be considered as truly representative of a novel antibiotic class. The last such compounds were oxazolidinones, with linezolid being introduced in 1996. Unfortunately, enterococcal resistance emerged shortly after it was put into clinical use (see **Figure 1**) [29]. As a result of the increasingly problematic drug resistance, some "forgotten" antibiotics have returned to clinical practice, such as colistin (polymyxin E), which is being used as a "drug of last resort" against Gram-negative bacteria [33]. It was discovered in 1950 by a group of Japanese scientists [24] but was abandoned shortly afterwards due to its nephrotoxicity [34] and the abundance of other equally potent antibiotics with less pronounced side effects. However, there have recently been reported cases of resistance which are hindering its effectiveness [35]. In 2016, plasmid-mediated dissemination of the *mcr-1* gene was reported in Escherichia [31] and only 2 years later the *mcr-8* gene was reported in Klebsiella [30] (see **Figure 1**).

It is therefore evident that novel compounds are urgently required to battle the ever evolving, multi-drug resistant bacteria. In 2017, the World Health Organization (WHO) made a list of top

priority strains for which new drugs are urgently or "critically" needed, including carbapenem-resistant *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and Enterobacteriaceae (together with 3rd generation cephalosporin-resistant ones) [36]. Research groups worldwide are now devoted to solving this crisis and among others [37,38], antimicrobial peptides (AMPs) are being considered as possible alternatives to classical antibiotic treatment [39].

2 Antimicrobial peptides – what are they?

"*I like the dreams of the future better than the history of the past.*"

-Thomas Jefferson (1743 - 1826)

There is no one-sided answer to this question, but somewhat of a consensus of what the antimicrobial peptides research community says that they (AMPs) are; *multifunctional effector molecules, produced by almost all organisms, having a direct antimicrobial activity and/or immunomodulatory properties* [40,41]. In simple terms, considering the constantly growing problem of antimicrobial resistance, and taking from the quote by Thomas Jefferson, AMPs could be "the dreams of the future". They are known to be variously active against Gram-negative and Gram-positive bacteria, viruses and fungi and sometimes also to exert antiparasitic and antiprotozoal properties [42]. On the other hand, they have been shown to modulate host immunity by activating immunocytes, suppressing inflammation and/or promoting auto-inflammation [43]. To emphasize their pleiotropic nature in higher organisms, natural AMPs are referred to as '*host defense peptides*' (HDP), or more specifically as '*innate defense regulatory* (IDR) peptides', since reports on their immunomodulatory activities have mostly been confirmed at the level of innate immunity [43,44]. Their impact and importance in innate defenses is supported by their abundance and distribution in all multicellular organisms including fungi, algae, plants, and invertebrate and vertebrate animals (see **Figure 2**). Up to now, the dedicated $CAMP_{R3}$ database contains entries for 8164 peptides the majority of which (~74%) have been identified in animals [45]. A particularly abundant source are anuran species, with almost two thousand peptide sequences reported in Database of Anuran Defense Peptides (DADP) [46]. AMPs are also present in prokaryotes, with bacteriocins being the most intensely studied. In these, however, their role is quite different to that in eukaryotes, and as "ancient weapons" are mainly used to clear competition by killing or inhibiting other, often closely related, bacterial strains [47].

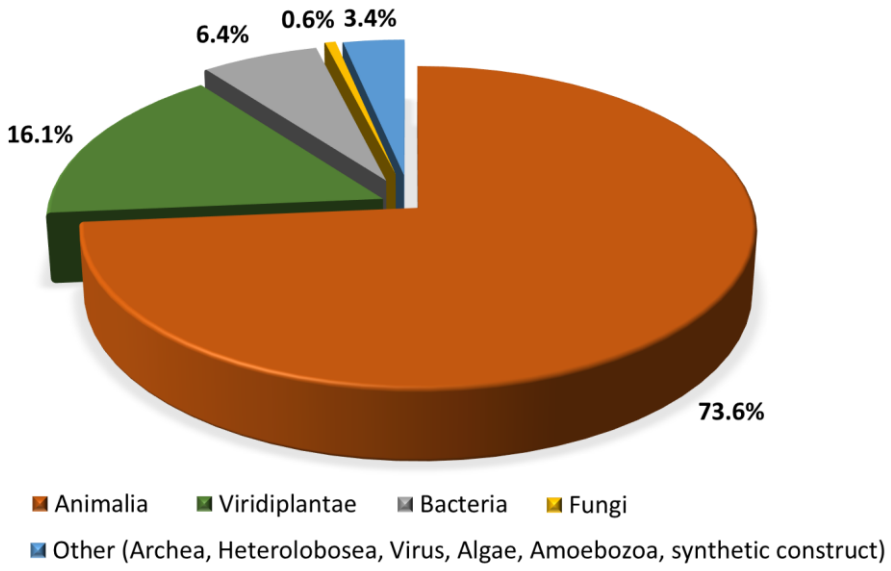

Figure 2. Distribution of AMPs across kingdoms based on sequences in the CAMP^{R3} database. (http://www.camp.bicnirrh.res.in/dbStat.php) [45].

2.1 Ribosomal vs non-ribosomal synthesis and peptide precursors

AMPs can be gene encoded and synthesized by mRNA translation on ribosomes or be assembled by large multifunctional enzymes known as nonribosomal peptide synthetases (NRPSs) [48,49]. The latter mechanism is used by bacteria and fungi [49] to incorporate both proteinogenic and non-proteinogenic amino acids into peptides, sometimes in the *D* configuration, and further modifying the peptides with ring formation, glycosylation, hydroxylation or acylation [50,51]. There are ~500 non-proteinogenic amino acids known as of today, which in contrast to only 20 proteinogenic amino acids, may possess added structural and functional features that can contribute significantly to a peptide's potency. In fact, the peptide antibiotics polymyxin B, gramicidin S and vancomycin, which are synthesized in this manner [51], all contain non-proteinogenic amino acids in part of their sequences [50,52].

Gene encoded, ribosomally synthesized peptides are produced by almost all forms of life, including bacteria [45,53,54]. Quite often, multiple AMPs are expressed at a single site due to clustering of co-regulated genes, as is the case with human α-defensins HNP1 and 4, HD5 and 6, and β-defensins hBD1 and 2 [55]. The peptides are also often expressed as inactive precursors, containing a signal peptide region and a negatively charged pro-piece that serve to convey the mature cationic peptide region to the site of action, where it is activated by their proteolytic removal (see **Figure 3**). In most cases these precede the AMP sequence, but in some

cases, such as for some fish and plant peptides, the prepro-sequence region can be located at the C-terminus [56,57].

The activity of AMPS is, therefore, regulated not only by their levels of expression but also by the presence and abundance of the appropriate proteases cleaving the peptide generally at dibasic cleavage sites [55,58]. The signal peptide is a common feature of prokaryotic and eukaryotic proteins, not only AMPs, allowing them to enter secretory pathways [59]. A very useful aspect is that signal regions of certain classes of antimicrobial peptides are highly evolutionary conserved (see **Figure 3**) [56,60,61], even though the mature peptides are highly divers [62]. The diversity of the mature AMP sequence most likely occurs as species' adaptation to specific microbial communities in a particular environment. On the other hand, while there is still no solid explanation for the phenomenon of signal sequence conservation, it gives valuable insights into the evolution of some AMP families, as is the case with the anuran peptides [63].

Finally, it is worth noting that a majority of gene encoded AMPs undergo post-translational modifications, currently classified into more than 15 types, including disulfide-bridge formations, N-terminal capping, halogenation, hydroxylation, phosphorylation, glycosylation etc. Peptides are modified to a greater or lesser extent, thus contributing to peptide potency and/or stability [64].

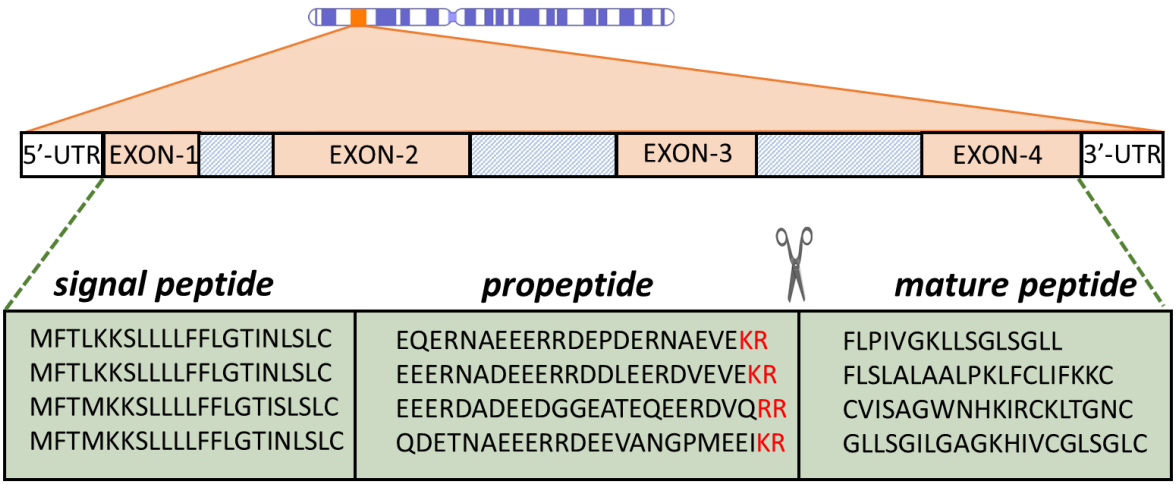

Figure 3. Schematic representation of AMP expression. Peptides are cleaved at dibasic cleavage site (-KR, -RR, in red). Depicted peptide sequences belong to anuran AMPs from Ranidae family [65–68].

2.2 Physico-chemical properties

There is no doubt that certain physico-chemical properties of AMPs and those of lipid molecules making up the bilayer system in the membrane of the target cells directly correlate with the peptides' biological activity and specificity. However, there is still an imperfect understanding of the complex relationship between the two, since even peptides with quite similar structures can have remarkably different reported mechanisms of action (e.g. buforin and magainin 2) [69]. A better understanding of the relationship is therefore required to elucidate those crucial features that are responsible for potency and specificity of AMPs.

2.2.1 Structure and length

AMPs can be divided into several categories depending on features of their secondary structure. The simplest classification divides them into extended structures, linear α-helical peptides and peptides with β-sheet or hairpin-like structures [70]. Guha et al. [71] have proposed a more elaborate taxonomy with 6 different classes including *i)* α-helix, *ii)*3/10 helix, *iii)* pi-helix, *iv)* β-strand, *v)* β-turn and *vi)* disorded coil, which may concern the entire peptide or only parts of it, with some scaffolds combining different structural motifs. Essentially, however, this classification still depicts three major classes of AMPs: helical, β-sheet and extended peptides. In this thesis, the latest Wang terminology will be used to classify peptides [72] in 4 classes: α-helical (e.g. human LL-37), β-sheet (e.g. human α-defensin-4), αβ-peptides (e.g. human β-defensin-2) and non-αβ-peptides (e.g. indolicidin) (see **Figure 4**).

Interestingly, α-helical peptides generally exhibit little or no structuring in polar settings, such as in bulk aqueous solution, and only adopt a defined secondary conformation in the presence of bacterial membrane or some other anisotropic environment (for example SDS micelles or a mixture of water and trifluoroethanol) [43]. Structuring is aided by *i*) the presence of helix-stabilizing residues (e.g., Leu, Ala, Lys); *ii*) salt-bridging between oppositely charged residues placed close to each other in the helix, i.e. at $i \pm 3$ or $i \pm 4$ positions and *iii*) interaction of hydrophobic residues with phospholipids in the membrane bilayer [73]. It should be noted that increasing helix stability can result in an increased potency, but only to a certain extent [74]. If it occurs to the extent that helix starts forming in bulk solution, the resulting aggregation of the so-formed amphipathic structures can negatively affect potency [75].

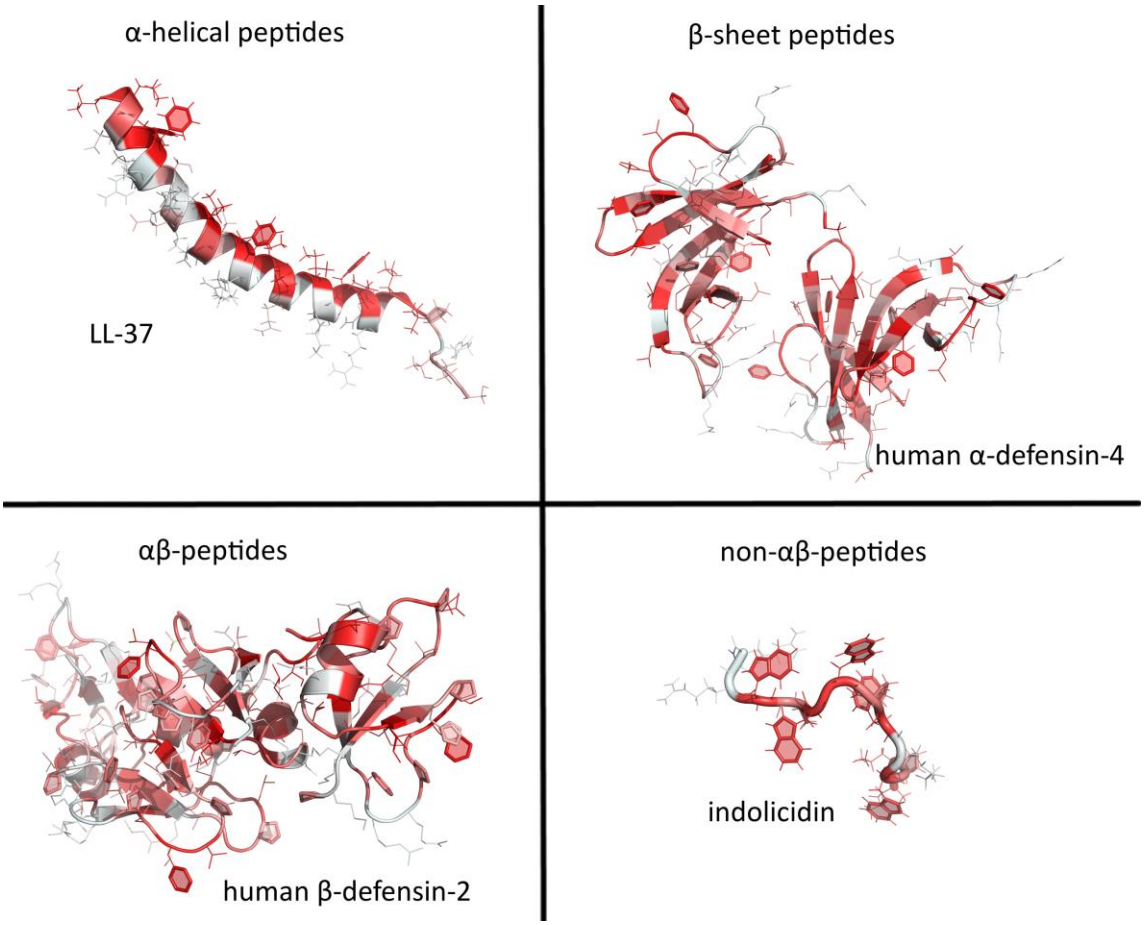

Figure 4. An overview of major structural classes of antimicrobial peptides. The above structures were solved either with NMR spectroscopy or X-ray diffraction and downloaded from Protein Data Bank (PDB) (https://www.rcsb.org/) [76]. PDB IDs: LL-37 (2k6o), human α-defensin-4 (1zmm), human β-defensin-2 (1fd3) and indolicidin (1g89). Visualization was done using PyMOL 1.8 [77] and amino acids colored according to normalized Eisenberg hydrophobicity scale (white – polar, red - hydrophobic) [78].

The length of the peptide is an important feature, especially in the context of peptide activity, since at least 7 – 8 amino acids are needed to form an amphipathic structure [69] with separate hydrophobic and hydrophilic faces. One of the shortest known peptides reported to have antibacterial activity, PGLa-H isolated from the skin of the African clawed frog *Xenopus laevis*, is only 10 residues long and adopts an α-helical conformation [79]. The peptide length is particularly important for those helical peptides that act by forming a barrel-stave pore (see below), as they have to transverse the entire lipid bilayer of the bacterial membrane, which requires at least 22 resides in a helical conformation, whereas the more extended β-sheet peptides should contain a minimum of only eight residues [69].

A peptide's length can also affect its cytotoxicity. Melittin shortened to just its 15 C-terminal residues is less toxic compared to the original 26-residue sequence [80], but this may be related to the removal of a section with features favoring toxicity, rather than just reducing the size. In fact, doubling the size of the 10-residue PGLa-H frog peptide in a tandem repeat resulted in a peptide with greater potency against bacteria without affecting the low toxicity towards host cells [81]. Therefore, it would be wrong to conclude that simple shortening or extending of a certain sequence results with a peptide with more favorable cytotoxicity properties. It may be more appropriate to use a sliding window to optimize the balance between antibacterial activity and host cell toxicity. In any case, natural AMPs generally have relatively short sequences (normally well under 100 amino residues), and the majority of known peptides are 10-30 residues long [42].

2.2.2 Charge and hydrophobicity

The net charge of known natural AMPs varies widely from anionic to cationic, ranging from -6 to +16 [73,82–84], but the vast majority of identified peptides have a net positive charge. For most peptides it falls in an intermediate range and it can be directly correlated with peptide potency and selectivity. There seems to be an optimum charge span for activity, so that both higher and lower values outside this range result in reduced activity and/or greater toxicity. Dathe et al. [85] have shown that increasing the charge of magainin analogs above +5 resulted in an increase of toxicity towards red blood cells and loss of antimicrobial potency. Giangaspero et al. [74] came to a similar conclusion when varying the charge of helical peptides which otherwise had relatively similar mean hydrophobicity, amphipathicity and helicity. In part, this effect was attributed to a reduced propensity for helix formation, due to the increased charge density. More recent findings suggest that it could also result from repulsion between neighboring peptides having a high net charge, resulting in a lower concentration of peptides adsorbed onto the lipid bilayer [86]. Finally, it should be considered that in principle the distribution of positively charged residues should not correlate with peptide potency, which should only depend on the overall peptide charge [74,87]. However, it can make a significant difference if it affects formation of helix-stabilizing salt bridges, as observed in artificial variants of the human helical peptide LL-37 [88].

In a similar way to charge, there seems to be an optimal hydrophobicity window for peptides to have a well-balanced ratio of antimicrobial activity and host cell toxicity. On average, AMPs contain approximately 50% hydrophobic residues. The overall hydrophobicity affects the peptide's capacity to partition into the lipid bilayer, and so can be directly correlated with both

antibacterial activity [89], but also host cell toxicity. Increasing or decreasing this property of the peptide outside an optimal range can respectively result in a rapid decrease of antimicrobial activity and an increased lysis of blood cells, not necessarily accompanied by improved antimicrobial activity [74,89,90]. In fact, a reduced antibacterial potency can be observed in peptides with higher hydrophobicity which can be attributed to peptide self-association and can also occur with excessive stabilization of the helical structure (see above). This can impede access to the bacterial membrane and therefore lower the concentration of peptide actually impacting on it [87,90].

2.2.3 Amphipathicity and helicity

Overall hydrophobicity is one of the key properties related with biological activity of any given AMP sequence. Unlike charge, where the distribution is not necessarily correlated with peptide potency (see above), the arrangement of hydrophobic residues (~ 50% in AMPs) and polar residues into an amphipathic structure plays a key roles in peptide activity, and is more importantly then the hydrophobicity itself [91]. But what exactly is the amphipathicity? This property refers to the topographic distribution of hydrophobic and polar residues within the peptide sequence, which results in a more or less accentuated spatial separation in the final AMP structure. For a helical conformation this results in polar/charged and hydrophobic residues clustering on opposite sides of the helix cylinder, and it can be numerically quantified in terms of the hydrophobic moment (μH) (see how this relates to the structure in the human helical AMP LL-37, in **Figure 5**) [78]. An α-helix is one of the simplest and more efficient ways to generate an amphipathic structure, although it is worth noting that β-sheet peptides can also possess an amphipathic arrangement to a greater or lesser extent, but it is more difficult to quantify [73,92]. Amphipathicity aids activity as it permits the helical structure to sink its hydrophobic face into the membrane bilayer, an essential step leading to membrane disruption. However, in this case too, it must be well tuned for an optimal balance between antibacterial potency and host cell toxicity. In general, the hydrophobic moment in helical AMPs is around 60% of the maximum possible value. Increasing it above this value does not greatly increase potency but can significantly increase toxicity [74,75].

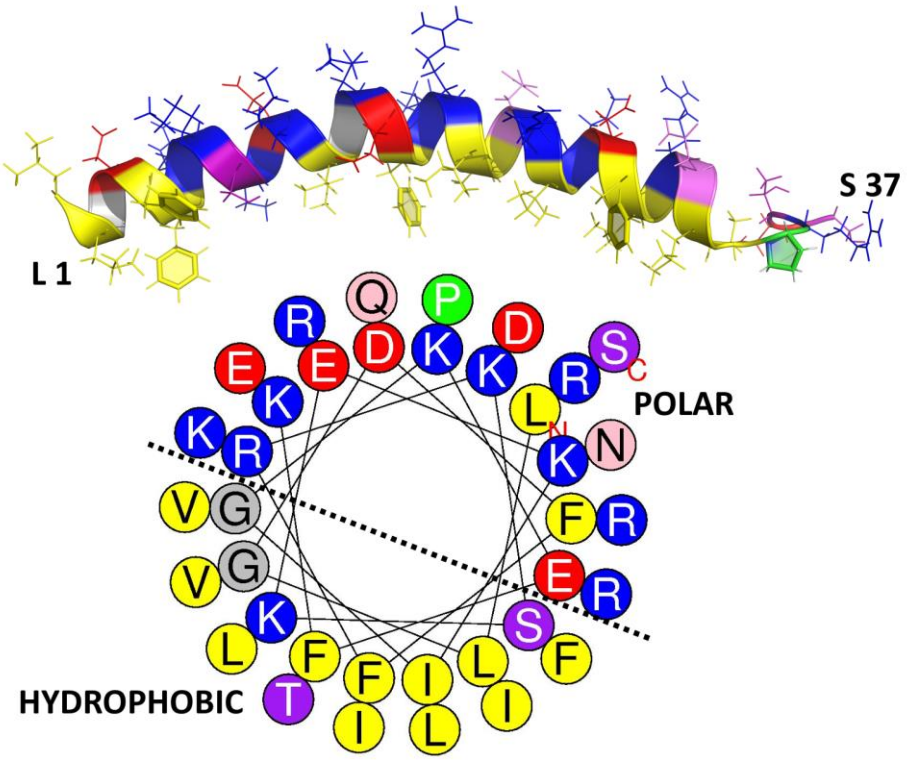

Figure 5. Secondary structure and helical wheel projection of human cathelicidin LL-37. The structure and projection were respectively obtained from PDB [73] (ID: 2k6o) and HeliQuest [93], respectively. The residues were colored according to their hydrophobicity with 38% hydrophobic and 62% polar amino acids in an appreciable amphipathic arrangement. Hydrophobic (yellow and green), polar charged [red (-) and blue (+)], polar uncharged (light to dark purple), glycine (grey).

Finally, helicity is the propensity of an AMP to adopt a helical structure. It is maybe not so crucial for a peptide's antibacterial activity as other factors discussed above, but it has been correlated with the toxicity towards host cells. It can be reduced by incorporating *D*-amino acids into the peptide sequence, without greatly affecting potency, although this can narrow the activity. As reported by Papo et al [94], replacing 35% of *L*-amino acids with their *D*-enantiomers resulted in peptides devoid of haemolytic activity that maintained an appreciable antibacterial potency. Furthermore, such structures are protected from proteolytic degradation, which should increase bioavailability of such synthetic peptides.

2.3 Mode of action

In the past decades, the mechanism of action of numerous AMPs has been extensively studied. These experiments have often been carried out on artificial membranes, typically large or giant unilamellar vesicles, and less frequently *in vivo* on microbial cells, using fluorescent

dyes and labeled peptides. In any case, it is generally accepted that AMPs can be categorized into *i*) membrane permeabilizing or *ii*) non-lytic peptides [95]. It is worth noting, however, that some peptides may act upon bacteria using either of these two major mechanisms, and sometimes both, depending on the concentration of AMPs and bacterial species or growth phase [96].

2.3.1 Membrane permeabilizing peptides (MPPs)

The term "membrane permeabilizing peptides" [71] is a rather more general one compared to the often used "pore-forming" peptides [97–99]. However, considering the complexity the lytic mechanism/s of membrane-active AMPs (which is not restricted only to "pore-forming"), it is a more accurate one (see **Figure 6**). For a peptide to be branded as an MPP, it must partition into a membrane and therefore be amphipathic for at least part of its structure, or simply put it, it must have some form of "interfacial activity". Such peptides can be extremely diverse structurally and can be active only against specific microbial species or have quite a broad spectrum of activity. Wimley's group has recently pointed out that the process of membrane permeabilization should not be considered as being simply due to stochastic events involving one or more peptide molecules, nor should it be ascribed to a well-defined sequence of events; in other words, it is neither a discrete nor static entity. Rather, it can be better described as a "mechanistic landscape" which varies depending on the experimental conditions and variables such as peptide concentration, bilayer lipid composition, temperature, ionic strength and pH [71]. It follows that even the molecular mechanisms of membrane permeabilization by well-studied AMPs have not been completely elucidated, and many questions remain to be answered [71,100]. This being said, certain common and well defined steps usually occur at the bacterial membrane, eventually leading to disruption: *i*) initial attraction of the AMP to the membrane surface, interaction and peptide structuring, *ii*) insertion and concentration dependent accumulation and *iii*) (in some cases) self-association/oligomerization [95], which may occur before or after step *ii)*.

Most α-helical peptides do not adopt this conformation in bulk solution (see above) so that the first interactions occur between a positively charged peptide coil and the negatively charged phospholipid head-groups in the bacterial membrane bilayer [73,101]. The initial adsorption onto the membrane therefore depends on electrostatic interactions, which allow hydrophobic interactions of a polar side-chains with the membrane acyl chains, which in turn induces structural changes in peptide (to a stable α-helical conformation) [102], which then inserts more

deeply into the lipid bilayer, altering its structure. By contrast, β-sheet peptides are stabilized by disulphide bridges that predispose an amphipathic residue arrangement. These peptides maintain the same stable conformation upon interaction with, and insertion into, the membrane.

Once on the membrane, a critical peptide concentration is needed to induce membrane lysis, which can occur by different mechanisms (see **Figure 6**). Some peptides also exhibit self-association and/or oligomerization at the membrane surface, before membrane perturbation occurs. This allows them to build up complex structures associated with their particular mode of action. For example, this behavior seems to be typical for human cathelicidin LL-37 [103] and alpha-defensin-6 [104]. After reaching threshold concentration (and possibly self-associating), several different mechanisms may explain the formation of membrane lesions, which can be more or less well defined entities:

i) peptides reorient from a parallel arrangement with respect to the membrane surface to a perpendicular orientation forming barrel-stave pores. This relatively rare mechanism is the case for alamethicin [105];

ii) peptides concentrate on and coat the surface until they form micellar structures involving small areas of lipid bilayer, which on removal from the membrane leaves a lesion. This detergent-like mechanism is also known as the carpet model and is typical for peptides such as magainin at higher concentrations [106];

iii) peptides align perpendicularly into the lipid bilayer with the hydrophobic region inserted in among the acyl chains, and the bilayer itself cavitates so that the hydrophilic region of the peptide faces the center of a pore (toroidal pore model) This is typical for bee venom derived peptide melittin [107].

The last two mechanisms are not necessarily mutually exclusive, but may occur at different peptide concentrations, in a membrane-dependent manner. Other less disruptive mechanisms have also been proposed, and may include membrane thinning, depolarization or fusion, electroporation and targeting specific phospholipids [70,73,100] (see **Figure 6**).

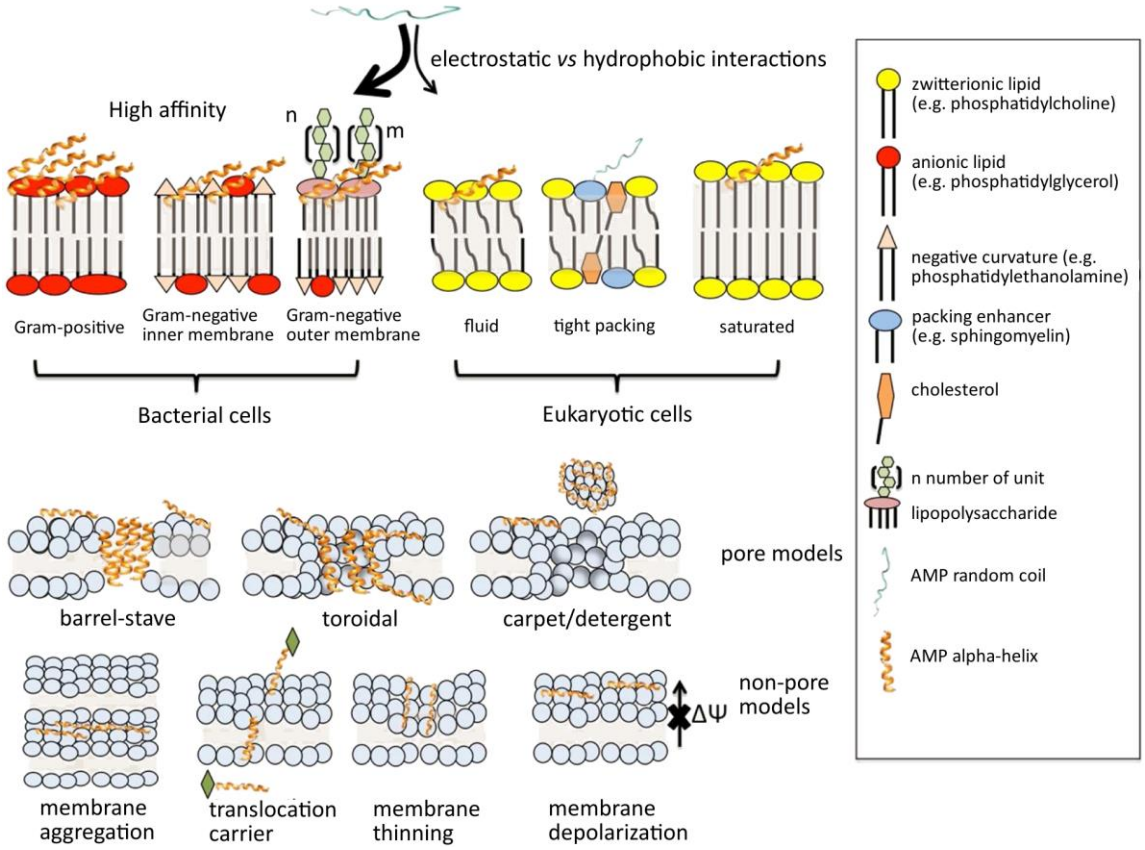

Figure 6. Proposed mechanisms of action of membrane permeabilizing peptides. Bacterial and eukaryotic cells are also represented to additionally pin-point their diversity and the complexity of peptide-lipid bilayer interaction [100]. Note that not all interactions include pore formation.

2.3.2 Non-lytic mechanisms of action

Some AMPs rely on more subtle approaches by traversing the bacterial membrane and reaching specific components inside the cell [70,96]. They can enter the cell either by direct penetration (a process in some ways comparable to pore formation) or by endocytosis, depending of their specific physico-chemical properties [108]. In some cases, the situation is not so straightforward, as is the case of proline-rich antimicrobial peptides, which enter the cell by a transporter-mediated process [109]. Regardless of the peptide uptake mechanism, these AMPs can be classified into several groups depending of their intracellular targets (see **Figure 7**) as listed below:

i) Nucleic acid biosynthesis and metabolism inhibitors

This group of peptides is represented by buforin II and indolicidin, respectively a proline-kinked helical peptide and extended, tryptophan rich peptide [110,111]. Buforin I, the parent peptide to buforin II (a 21 amino acid fragment) is homologues to the N-terminal fragment of the DNA-binding protein histone H2A [112]. Variants of the latter have shown the affinity towards double stranded nucleic acids [113], whereas in some cases the designed analogues (such as BF2-A) were found to have a greater binding affinity for RNA [114]. Similarly, indolicidin, a peptide of bovine origin belonging to the cathelicidin family, has been found to act by disrupting the bacterial membrane and by inhibiting DNA synthesis, more specifically inactivating DNA topoisomerase [111,115,116].

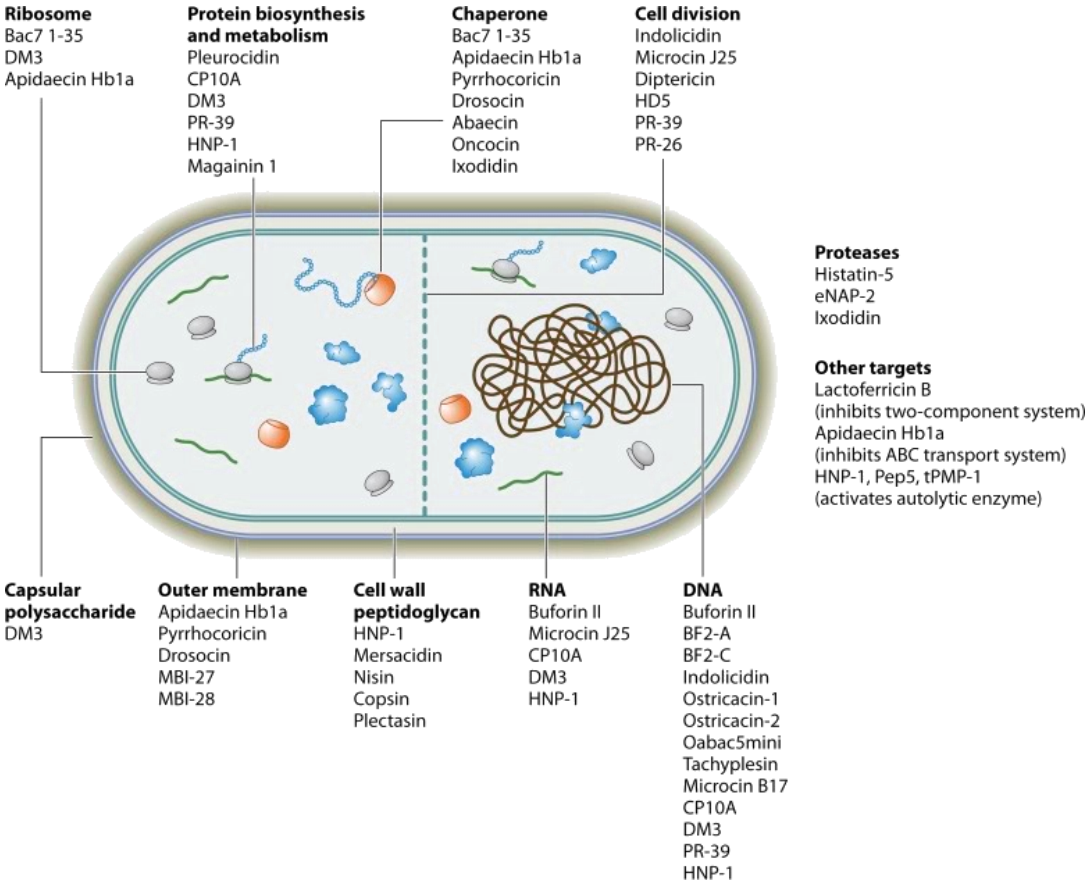

Figure 7. Schematic representation of major targets of non-lytic AMPs. Some peptides such as indolicidin and buforin II are inhibiting multiple intracellular targets [117].

ii) Protein biosynthesis and metabolism inhibitors

Bovine cathelicidin Bac7, a 60 amino acid long peptide isolated from neutrophils, interferes with complex machinery involved in protein synthesis. Its activity seems to reside at the N-terminus, so that a 35-long fragment, $Bac7_{1-35,}$ is fully active and has been shown to inhibit

protein translation by targeting the ribosome subunits [117,118], without affecting DNA synthesis and transcription, thus specifically inhibiting the process of protein synthesis [119]. Other proline rich peptides with a similar mode of action include PR-39, a cathelicidin peptide from porcine small intestine [120] and apidaecin-type peptides isolated in honeybees, hornets, and wasps [121]. Apart from Pro-rich peptides, CP10A, a synthetic indolicidin derivative in which proline has been substituted with alanine, is an example of a short, tryptophan-rich helical peptide that in addition to membranolytic properties has DNA-binding affinities, and also acts by disrupting protein metabolism [122].

iii) Protein-folding and protease inhibitors

A group of short, proline-rich and insect-derived peptides exert antimicrobial activities by interfering with protein-folding. Pyrrhocoricin, apidaecin and drosocin inhibit a major bacterial heat shock protein known as DnaK, and in some cases disrupt its ATPase activity [123–125]. Pyrrhocoricin and drosocin prevent DnaK from refolding misfolded proteins, whereas apidaecin has also been shown to inhibit the associated chaperonin GroEL [117,126]. All three AMPs bind stereospecifically, making them inactive towards the human counterpart chaperone Hsp70 [123]. Other peptides with the same mode of action include abaecin, oncocin and also the bovine cathelicidin-derived $Bac7_{1-35}$ [117]. Additionally, a plant peptide from *Medicago truncatula*, NCR247 also binds to GroEL, but the significance of this interaction hasn't been elucidated thus far [127].

Some peptides, like histatin-5 act against host- and bacterium-secreted proteases [128]. Dysregulation and overproduction of host- and bacterial proteases are associated with oral diseases, arthritis, periodontitis and tumor metastasis. Therefore, histatin 5 which competitively inhibits cysteine proteinase clostripain, produced by the anaerobic bacterium *Clostridium histolyticum* that causes gas gangrene [129], and other similar peptides, could serve as a potential therapeutic to reduce extracellular matrix degradation caused by dysregulated proteases.

iv) Cell division inhibitors

CRAMP, an amphipathic, α-helical cathelicidin identified in mouse and orthologous to human LL-37, is a potent membranolytic as expected of a helical AMP [130]. However, it also interferes with the septation process, most likely by inhibiting cytokinesis, although this has yet to be confirmed [126]. Human α-defensin 5 targets different cell mechanisms, but also

interferes with cell division processes as showed by extensive elongation of α-defensin 5 treated bacteria [131]. Other peptides, like microcin J25, diptericin, indolicidin and PR-39 also interfere with the process of cell division [117], however, there is still no strong evidence of the precise mechanisms involved.

v) Cell wall biosynthesis inhibitors

The bacterial cell wall provides structural integrity to the cell and plays a key roles in survival of bacteria. For this reason it is a prominent target for numerous antibiotics in use today [132]. It consists of alternating β-1,4-linked N-acetylglucosamine and N-acetylmuramic acid crosslinked with peptide chains [133]. Lipid II is an important component of the cell wall synthesis process, acting as shuttle carrier transporting N-acetylmuramic acid across the membrane to be incorporated into existing cell wall structure [134]. Considering the cell wall is not present in eukaryotic cells it provides reasonable target for therapeutic applications. A number of AMPs including lantibiotic peptides mersacidin [135] and nisin (also acts by pore formation) [136], and fungal defensins plectasin and copsin [137,138] act in this way.

vi) Lipopolysaccharide (LPS)-binding peptides

These peptides specifically act against Gram-negative bacteria since LPS is a major structural and functional component of the outer membrane of Gram-negatives and covers most of their surface. LPS can be released during bacterial cell division or death and induce a variety of inflammatory effects in humans. Antibiotics used to clear Gram-negative infections may cause the release of such molecules, leading to sepsis, and at the moment there is limited treatment for patients with septic shock, which most often results in death [139,140].

The LPS layer can actively neutralize the activity of AMPs by inducing their self-association or aggregation and sequestering them [141]. This has been observed for the frog peptides temporins A and B from *Rana temporaria*. However, Rosenfeld et al. [142] showed a synergic effect between these peptides and temporin L, which prevents their LPS-mediated oligomerization and markedly improves their activity. Another way to restore activity is the introduction of a so-called boomerang motif (GWKRKRFG) at their C-terminus resulting in hybrid peptides no longer susceptible to LPS-induced aggregation [143]. Furthermore, melittin-cecropin hybrid peptide with two additional positive charges at C-terminus proved to be effective in traversing the LPS layer [144]. In any case, amphipathicity and a high proportion of cationic residues in the AMP sequence seem to be important properties for the broad-spectrum LPS-binding peptides [145].

2.4 Strategies for identification and design

2.4.1 Crude but effective – extraction and assay-guided isolation

In the past, identification of novel AMPs involved handling of several specimens from the same species to obtain small amounts of active peptides. Initial tissue homogenization was followed by peptide extraction and the crude peptide was isolated in several steps, mainly using chromatographic techniques. In some cases, the animals were pretreated with electric shocks or noradrenaline to stimulate AMP production, or exposing the organism to bacterial infection, before isolation [146,147]. Potential AMPs were then identified by antimicrobial activity testing of the obtained fractions and, finally, the sequence was determined using different techniques, including mass spectrometry. Magainin, one of the first identified frog peptides, was isolated in this manner [148], and a similar procedure was used for the identification of penaeidins, pleurocidin and some mollusk cysteine-rich peptides, among others [149–151]. Additionally, some human peptides from epithelial cells and plasma were also identified in the same way [152–155]. The described approach is evidently successful, but also very time-consuming, and with low yields. It can also raise ethical questions of animal protection, especially considering rare and endangered species. Finally, many AMPs can be missed by this approach, if they are not constitutively produced and their expression can't be stimulated as described.

2.4.2 Abundance of omics data – can we make the most of it?

The development of sequencing techniques opened the possibility of mining for valuable information otherwise hidden in the genome, without the necessity of isolating and analyzing polypeptides. Frog temporins have been identified by isolating total RNA and reverse transcribing the mRNA based on the 3' poly-A tail. A cDNA library was constructed by using appropriate vectors and the positive clones selected and analyzed by nucleotide sequencing. This gave information about several novel AMPs and the results were then verified using "classical methods" including isolation, purification, activity testing and amino acid analyses [147]. A similar procedure led to discovery of several peptides in the pickerel frog, *Rana palustris* [156], of clavanins from tunicate hemocytes [157], of protegrins from porcine leukocytes [158] and of penaeidins from Indian white shrimp *Fenneropenaeus indicus* [159], among others. More recently, *in silico* analyses of cDNA data in EST databse [160] led to discovery of trichoplaxin, a placozoan AMP from *Trichoplax adhaerens* [161]. Improvement

of next-generation sequencing techniques and analysis pipelines, as well as the abundance of publicly available genomic and transcriptomic data, led to the development of high-throughput techniques for simultaneous identification of potential AMPs. Kim et al. [162] reported a *de novo* transcriptome analysis of the American cockroach *Periplaneta Americana*, leading to the discovery of 86 antimicrobial peptides out of which 21 were experimentally verified for activity. A similar approach was used for identification of novel AMP sequences in the grasshopper *Oxya chinensis sinuosa* [163]. Yi et al. [164], on the other hand, screened seven previously assembled genomic and transcriptomic datasets in the amphibious mudskippers and, based on sequence similarity, identified ~500 novel peptide sequences opening new pathways for AMP discovery. The process of identifying putative AMP sequences from nucleic acid databases is in any case essentially knowledge-based; it requires having information about a relatively well-conserved sequence in at least a part of a given class of AMPs, and/or conserved motifs or physico-chemical features that confer antimicrobial activity.

2.4.3 Quantitative structure-activity relationship (QSAR) studies

The above-mentioned methods have proven to be effective in identifying putative AMPs but cannot predict the potency of their activity towards bacteria and/or their toxicity towards host cells. Rational design of artificial peptides or redesign of natural peptides, based on varying physico-chemical properties associated with potency and/or selectivity (e.g. net charge, amphipathicity, structuring propensity, tendency for self-aggregation etc.), has to a certain extent provided data that can also be used in predicting functional characteristics, especially, but not only, for linear helical peptides [165,166]. Other approaches may include virtual screening studies where peptide biophysical properties are used to construct molecular descriptors associated with different functional aspects. Based on quantitative structure-activity relationship (QSAR) modelling, these descriptors are then used to link a sequence to its likely biological activity [167]. Adepantins were designed in this way, based on descriptors extracted from frog AMPs with measured potency against *E. coli* and hemolytic activity, and proven to be remarkably selective towards some Gram-negative species [168]. Such predictors were based on 2D-models, but more recently, 3D-descriptors have also been developed [169,170]. This has, in part, been possible due to molecular dynamics (MD) simulations, which were used for the optimization of the starting 3D structure models since the number of experimentally determined 3D structures is still rather limited [170]. Although they always have to be considered critically, and eventually verified experimentally, MD simulations have the ability to go “beyond the experiment”, giving valuable information at the atomistic level. They can

provide considerable insight on the mode of action of some peptides, thus improving the existing models used for AMP prediction [171].

2.5 Therapeutic potential

Since their initial discovery, AMPs have often been discussed as potential novel therapeutic agents for the treatment of bacterial, fungal and parasitic infections. Indeed, this has been one of the driving forces behind AMP research. However, translational applications of such peptides, and the transition from *in vitro* to *in vivo* have proven to be quite difficult [172,173]. Compared to "classical" antibiotic treatment, AMPs should have several advantages, since they are multimodal (can hit different bacterial targets simultaneously), multifunctional (can directly inactivate microbes but also stimulate defence against them), fast acting, often bactericidal and can have accessory anti-inflammatory and healing activities. [174]. However, they have evolved to act in a precisely orchestrated manner and are difficult to deliver as exogenous drugs. Furthermore, bacteria can fight back and interfere with AMP activity through proteolytic processing, active efflux, biofilm formation and exopolymers entrapment, as well as by reducing their surface charge [175–177]. This being said, AMPs generally elicit low levels of resistance compared to "classical" antibiotics, which are expensive for the bacteria to maintain, so temporary.

Despite years of trials, there are still some major obstacles to overcome before possible clinical applications of AMPs. Potent antimicrobial activity is often accompanied by toxicity towards host cells. Although cationic AMPs preferably target the negatively charged bacterial membrane, it has proven to be difficult to completely eliminate toxicity towards host cells, and it can be significant for amphipathic, helical AMPs. Melittin, for instance, is cytotoxic at comparable concentrations conferring antimicrobial activity [178]. Furthermore, AMPs, are often toxic towards the indigenous microflora, which can cause additional complications by removing its protective function [179]. These issues can in principle be resolved by designing peptides with favorable physico-chemical properties (see above). Brevinin-1EMa analogues were less haemolytic when Ala residues replaced Leu, to reduce hydrophobicity [180] and a similar effect was observed when hydrophobicity was reduced at the N-terminus of mastoparan-X peptides [181]. In general, however, there is a trade-off between reduced toxicity and reduced potency. Alternatively, other approaches used for reducing peptide toxicity may include nanoencapsulation, as was the case with P34 [182].

A second major concern is peptide stability/bioavailability under physiological conditions (i.e. in the presence of serum, salt, pH variations, proteolytic enzymes) that can result in unfavorable pharmacokinetics. This is especially the case with linear peptides, which are easily attacked by host proteases and peptidases [179], and particularly problematic if the peptides are administered systemically [183]. For this reason, AMPs are predominantly being considered for topical applications. The stability can, however, be enhanced by peptide cyclization (linking the C- and N-terminus) preventing the proteases interaction with the liner peptide due to steric hindrance [184]. Another possible solution is to introduce *D*-isomers or unnatural amino acids into the peptide sequence, making it unfit for enzyme degradation [185]. However, this is expensive and precludes biosynthesis. Alternatively, nanoencapsulation can improve peptide stability while at the same time reducing toxicity [182]. Another strategy is to PEGylate peptides (link them to polyethylene glycol), which has also been shown to increase bioavailability by reducing renal clearance [186].

Finally, high manufacturing costs represent another major obstacle for peptide antibiotics [51,179]. Production of one gram of such drug by means of solid phase chemical synthesis can cost several hundred dollars. Therefore, there is a need for less expensive production platforms such as, for instance, fungal or bacterial expression system. In recent years, several attempts have been made in this field, however, none proved to be commercially feasible. An exception is the fungal expression system which was successful in obtaining sufficient amounts of plectasin [187].

Despite these obstacles, approximately twenty AMPs are in various stages of clinical trials at the moment, with the majority intended for topical applications [188]. They include cyclic and linear AMPs, such as a twelve residue histatin derivative, P-113, the magainin derivative MSI-78 (pexiganan, which however failed to gain FDA approval for its original use); the twelve residue indolicidin derivative omiganan; the arenicin-3 analog AA139; the cyclic protegrin I analog murepavadin, and others [179,188].

II AIMS AND SCOPES OF THE THESIS

The first objective of this research was to develop a new methodology for simultaneous identification of novel AMPs from multiple anuran species, exploiting the highly conserved signal peptide region of AMP precursors, to scan available omics data, and then use the sequences to design primers to guide high-throughput sequencing. For this purpose, peptides could be identified by selectively amplifying AMP-coding transcripts from very small amounts of frog skin tissue samples. This required collection of frog species pertaining to three different families - Ranidae, Hylidae and Bombinatoridae. Simultaneously, conserved signal peptide regions were obtained from the Database of Anuran Defense Peptides (DADP) [189] while available RNAseq data for anuran species belonging to the same families was retrieved from the Sequence Read Archive database [190]. The signal peptide sequences from DADP were aligned and used to generate Hidden Markov Model (HMM) profiles [191]. The assembled RNAseq transcripts were translated to all six possible reading frames and then screened for significant matches with HMM profile. Open reading frames (ORFs) encoding putative AMPs, which corresponded to positive hits, were used to design family specific forward degenerate primers. Total RNA was then extracted from the frog tissues, and cDNA was prepared using reverse primer based on the mRNA poly-A tail. This was followed by PCR amplification and high-throughput sequencing of the size-selected amplicons. After trimming, transcripts were blasted against a custom built peptide database to detect all positive hits, and the longest ORFs of the resulting contigs translated into amino acid sequences [192].

A second objective of the proposed research was to design novel peptides by implementing subtle changes to previously identified sequences of anuran origin. Such modifications can be effected based either on prior knowledge, taking into consideration relevant physico-chemical properties (e.g. net charge, amphipathicity, tendency for self-aggregation) associated with peptide activity and/or selectivity, or using the available QSAR tools such as Mutator [193], in order to increase AMP selectivity by introducing one or two amino acid substitutions into parent peptide sequence. Based on the requirements of the algorithm, the selectivity index (SI) can be increased either by decreasing peptide toxicity (i.e. increase the HC_{50} values), or by increasing antibacterial potency (i.e. decreasing the MIC values) while not altering the toxicity. For this purpose, and instead of inputting peptides ‘one by one’, a filtering algorithm has been implemented on the DADP database to first extract peptide sequences based on a threshold MIC value against a specific bacterial strain. The filtering algorithm was then used to extract peptides with desirable characteristics, namely a specified peptide length and appropriate

theoretical SI values. This resulted in a subset of peptides which were inputted into the Mutator algorithm that suggested limited mutations to potentially increase the SI.

A final objective of this thesis was to evaluate the biological and biophysical properties of the novel peptides suggested by the two methods. For this purpose, eight peptides, either identified or re/designed, were extensively tested for their antimicrobial activity and toxicity. The peptides' structure and mode of action have also been evaluated with circular dichroism, flow cytometry, surface plasmon resonance and atomic force microscopy. The tested peptides varied significantly in structure and selectivity, whereas the mode of action indicated membrane perturbation to a greater or lesser extent for all the tested AMPs.

III DISCOVERY AND CHARACTERIZATION OF ANURAN AMPs

1 Parallel identification of novel antimicrobial peptide sequences from multiple anuran species by targeted DNA sequencing

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The main incentive for this paper, which served as a kick-off for the entire thesis, was to develop new methodologies for fast, reliable, high-throughput and simultaneous identification of novel AMPs from multiple anuran species. The invasiveness of some other well-established methods (see Introduction) arguably raises questions regarding animal protection, especially considering rare and/or endangered species, which is the case for many amphibian species. The suggested approach requires only small amounts of tissue to amplify AMP-coding transcripts, by exploiting the highly conserved character of available peptide precursor regions (i.e. signal peptide and propeptide regions). Given the fact that such data, together with RNAseq, are freely available in dedicated databases, they were used to construct family-specific forward primers. Total RNA extraction was then followed by cDNA synthesis, selectively targeting for AMP cDNAs amplification, and sequencing of the resulting amplicons. Using this method, >100 putative AMP sequences were identified in frog skin tissues, an abundant source of previously known peptides, out of which 29 sequences were novel. The results were encouraging *i*) since the method evidently served to identify previously unknown peptides, *ii*) the identification of ~100 previously known AMPs was an additional validation of the selected approach. This is particularly important considering that frog AMPs, especially from skin tissue, are well studied. It additionally suggested that the approach might be used as a "deep screening" method, capable of identifying peptides where other methods are lacking, taking into account, however, that the method's success depends on a number of conditions. In any case, the approach was evidently successful, and it can be used for AMP identification given the presence of conserved signal peptide and/or propeptide regions in other species as well, "as is", or after some suggested improvements.

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Parallel identification of novel antimicrobial peptide sequences from multiple anuran species by targeted DNA sequencing

Tomislav Rončević[1*], Marco Gerdol[2], Francesca Spazzali[2], Fiorella Florian[2], Stjepan Mekinić[3], Alessandro Tossi[2] and Alberto Pallavicini[2]

Abstract

Background: Antimicrobial peptides (AMPs) are multifunctional effector molecules that often combine direct antimicrobial activities with signaling or immunomodulatory functions. The skin secretions of anurans contain a variety of such bioactive peptides. The identification of AMPs from frog species often requires sacrificing several specimens to obtain small quantities of crude peptides, followed by activity based fractionation to identify the active principles.

Results: We report an efficient alternative approach to selectively amplify AMP-coding transcripts from very small amounts of tissue samples, based on RNA extraction and cDNA synthesis, followed by PCR amplification and high-throughput sequencing of size-selected amplicons. This protocol exploits the highly conserved signal peptide region of the AMP precursors from Ranidae, Hylidae and Bombinatoridae for the design of family-specific, forward degenerate primers, coupled with a reverse primer targeting the mRNA poly-A tail.

Conclusions: Analysis of the assembled sequencing output allowed to identify more than a hundred full-length mature peptides, mostly from Ranidae species, including several novel potential AMPs for functional characterization. This (i) confirms the effectiveness of the experimental approach and indicates points for protocol optimization to account for particular cases, and (ii) encourages the application of the same methodology to other multigenic AMP families, also from other genera, sharing common features as in anuran AMPs.

Keywords: Antimicrobial peptides, Anura, Innate immunity, Parallel identification, Signal peptide region

Background

AMPs are endogenous antibiotics present in all organisms, with a direct antimicrobial activity towards pathogens, often also showing immunomodulatory properties, and a regulated gene expression to facilitate and modulate immune responses [1, 2]. The skin secretions of many anurans contain a variety of bioactive peptides encoded by multigenic families [3] that often exhibit antibacterial activity towards multidrug resistant microbial isolates [4]. AMPs have been identified in all anuran families of the phylogenetically more ancient suborder of Archaeobatrachia including Leiopelmatidae, Alytidae, Bombinatoridae and Pipidae families. In Neobatrachia, AMPs have been identified in Dicroglossidae, Hylidae, Hyperoliidae, Leptodactylidae, Myobatrachidae and, in particular, in Ranidae [5]. The latter family consists of wide-ranging frog species distributed worldwide, except for the polar regions, in which 14 different AMP classes have been identified to date based on the molecular characteristics of the peptides they contain. Ranid frogs, in general, are well known to synthesize and secrete multiple active AMPs (at least 22 are reported in the skin secretions of *Rana palustris*) with rare exceptions such as in *Rana sylvatica*, in which only one antimicrobial peptide has been isolated to date [6]. It is worth noting that production of antimicrobial peptides may be influenced by hormonal and/or environmental factors [7–9], which can

* Correspondence: troncevic@pmfst.hr
[1]Department of Physics, Faculty of Science, University of Split, 21000 Split, Croatia
Full list of author information is available at the end of the article

hinder the identification of AMPs under certain experimental conditions.

Identifying novel anuran peptides normally requires handling several individuals, which are either sacrificed or held in captivity and treated with electric shocks/norepinephrine to obtain small amounts of crude peptide. This is followed by several rounds of purification using different precipitation and chromatographic techniques combined with activity testing of fractions to identify the active principles [10]. This approach however raises problems of animal protection and nature preservation. The International Union for Conservation of Nature (IUCN) reports that 1276 amphibian species worldwide are endangered or critically endangered with 38 having become extinct [11]. In this context, the search for a more efficient and less invasive method that requires minimum amounts of biological samples is highly desirable, such as isolation, amplification and sequencing of the nucleotide sequences coding for the AMPs. Although a few alternative approaches based on the screening of available transcriptomic data have been attempted [12], they did not implement the use of degenerate primers designed on the most conserved regions of AMP precursors [13]. This has resulted in the discovery of a limited number of novel AMPs and has never been applied for large-scale multispecies screening.

We have developed a potentially faster, less invasive and more efficient approach based on the selective amplification and subsequent sequencing of transcripts encoding for antimicrobial peptides, starting from very small amounts of tissue. This method however requires accurate primer design to capture the diversity of AMPs. In general, anuran antimicrobial peptide precursors consist of a highly conserved signal sequence, a negatively charged propeptide and a hypervariable cationic mature region [14–16]. Data on anuran signal sequences pertaining to different families and species are available in a dedicated database, DADP [17]. In many cases, the sequences present in this database were validated by biochemical methods, which also confirmed biological activity. Using this information and combining it with a method based on the 3'-RACE (rapid amplification of cDNA ends) protocol [18] we have developed a methodology for simultaneous identification of novel antimicrobial peptide sequences from multiple anuran species. To this purpose, total RNA was extracted from eight different frog species belonging to three anuran families. cDNA libraries were prepared utilizing a reverse primer based on the mRNA poly-A tail and forward degenerate primers designed based on highly conserved signal regions of the peptide precursors. These were used for selective amplification of the target AMP cDNAs, and the resulting amplicons then size-selected and subjected to Ion Torrent long-read high-throughput sequencing. We present data on the effectiveness of this method in identifying AMPs, including several known sequences and a number of novel sequences, some belonging to known classes. We also indicate deficiencies, discuss the most likely causes and indicate how to possibly overcome them.

Methods

Tissue sampling and RNA extraction

One specimen belonging to each of eight different species from Ranidae, Hylidae and Bombinatoridae family (see Additional file 1) was collected in the Croatian wild during March and April 2017. Frogs were sampled in accordance with applicable EU and Croatian legislation governing animal experimentation (Directive 2010/63/EU and NN 55/2013) and necessary permits were obtained from Croatian Ministry of Environmental and Nature Preservation. All animals were sacrificed by exposure to chloroform 24–48 h after capture to ensure a minimal stay in captivity and suffering. Approximately 200 mg of skin tissue was immediately transferred to RNA*later*® buffer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and stored at − 20 °C according to the manufacturers' instructions. Total RNA was extracted using the TRIzol protocol (TRIzol® Reagent, Life Technologies, Carlsbad, California, USA) from ~ 50 mg of this tissue, resuspended in RNAase free water, quantified with NanoDrop 2000 (Thermo Fisher Scientific, Waltham, Massachusetts, USA), quality checked using denaturing 1.5% agarose gel electrophoresis, and stored at − 80 °C until further use (see Fig. 1).

Transcriptome assembly and screening

The available RNAseq data in Sequence Read Archive database [19] was retrieved for 16 anuran species belonging to 3 different families, and assembled with the Trinity 2.4.0 software [20] (see Additional file 2) using default parameters and setting a minimal contig length to 200 nucleotides. Signal sequences pertaining to Class-1 (Ranidae and Hylidae) and Class-3 (Bombinatoridae) families were obtained from DADP database taking into account only peptides with reported bioactivity data [17] (see Additional file 3). The protein sequences from DADP were aligned using Muscle [21] and used to generate Hidden Markov Model profiles with the HMMER 3.1b1 hmmbuild module [22]. Trinity assembled transcripts were translated to all six possible reading frames with EMBOSS transeq [23, 24] and then screened for significant matches using the HMMER 3.1b1 hmmsearch module with an E-value cut off < 0.05. Open reading frames (ORFs) encoding

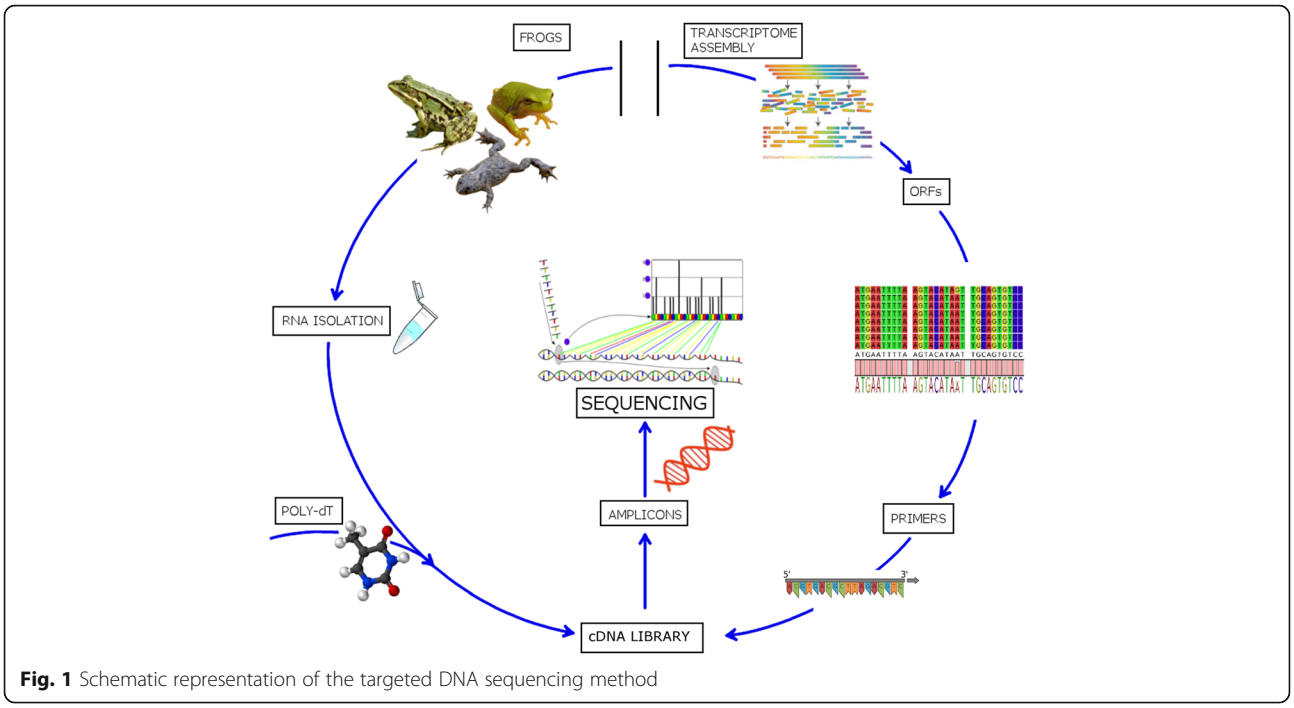

Fig. 1 Schematic representation of the targeted DNA sequencing method

peptides corresponding to positive hits were extracted from each transcriptome with CLC Genomics Workbench 10.1.1 (Qiagen, Hilden, Germany). Incomplete ORFs were also retained.

Primer design

The nucleotide sequences from Ranidae and Hylidae AMP transcripts obtained as described above were separately aligned and only the regions encoding for the signal peptides were kept for further analysis. Redundancies were removed and the remaining sequences were clustered by similarity with the cd-hit software, using a 0.8 identity threshold (i.e. all signal peptide sequences sharing > 50% sequence identity at the nucleotide level were clustered together) [25]. The same procedure was used for the Bombinatoridae family, but in this case longer highly conserved regions were obtained comprising the propeptide region. The resulting alignments of sequence clusters were used for forward primer design (see Additional file 4). Briefly, the position of forward primers was selected based on the identification of well-conserved 20 nucleotide-long sequence stretches containing a maximum of 3 polymorphic positions, where degenerate nucleotides were inserted (see Table 1). Due to the short length of the signal peptide region of Ranidae and Hylidae AMPs (about 60 nucleotides), the positioning of the forward degenerate primer was not expected to have a significant effect on amplicon size and therefore the maximum allowance of 3

Table 1 List of primers used for cDNA synthesis and amplification

Target family	Primer	Primer sequence (5′-3′)
Forward primers		
Ranidae[a]	TP1	CAGGACCAGGGTACGGTG**ATGTTCACCTTGAAGRAATC**
Ranidae[a]	TP2	CAGGACCAGGGTACGGTG**TTGGGATYGTCTCCTCATCT**
Ranidae[a]	TP3	CAGGACCAGGGTACGGTG**ATGTTCACCWTGARKAAAYC**
Hylidae[b]	TP4	CAGGACCAGGGTACGGTG**AAGAARTCWCTTYTTCTTGT**
Bombinatoridae[c]	TP5	CAGGACCAGGGTACGGTG**TAGAAGAAGAATCACTGAGG**
Outer PCR *Forward*	A-Uni1	CCATCTCATCCCTGCGTGTCTCCGACTCAG$(X)_{10}$CAGGACCAGGGTACGGTG
PCR *Reverse*	trP1	CCTCTCTATGGGCAGTCGGTGAT

AMP specific sequences are in bold
[a]Species pertaining to Ranidae family: *Pelophylax ridibundus*, *Pelophylax* kl. *esculentus*, *Rana dalmatina*, *Rana arvalis* and *Rana temporaria*
[b]Species pertaining to Hylidae family: *Hyla arborea*
[c]Species pertaining to Bombinatoridae family: *Bombina variegata* and *Bombina bombina*

polymorphic positions was aimed at minimizing the chances of non-specific PCR product amplification. In the case of Bombinatoridae, due to the different organization of AMP precursors, the forward primer was designed as close as possible to the 3'end of the propeptide-encoding region, maintaining the maximum limit of three degenerate nucleotides.

The reverse primer (5'-CCTCTCTATGGGCAGTCGG TGATTTTTTTTTTTTTTTTTTT-3′) contained a poly-dT stretch to match the poly-A tail of the mRNA and was used for cDNA synthesis. It also contained a 5′ tail sequence (trP1) which was required for the cDNA amplification protocol and subsequent parallel sequencing. All forward primers (see Table 1) were also synthesized with a 5'-CAGGACCAGGGTACGGTG-3′ tail required for multiplex sequencing through attachment of the barcodes in a secondary outer amplification. All primers were synthesized by BMR Genomics (Padova, Italy) (see Fig. 1).

Library construction and sequencing

First strand cDNA was synthesized from 1 μg of total RNA using the qScript™ Flex cDNA Synthesis Kit (Quanta Biosciences, Gaithersburg, Maryland, USA) according to the manufacturers' instructions. The reverse transcription reaction was performed at 37 °C for 1 h. The mixture for cDNA amplification contained 0.2 μl of DNA polymerase (5 U/μl), 2.5 μl of 25 mM $MgCl_2$, 2.5 μl of 10 × buffer A (KAPA Taq PCR Kit, Kapa Biosystems, Wilmington, Massachusetts, USA) together with 0.5 μl of 10 μM dNTP solution, 0.5 μl of 10 μM specific forward primer solution, 0.5 μl of 10 μM trP1 primer solution and 1 μl of cDNA template in a total volume of 20 μl. The PCR started with an initial denaturation at 95 °C for 2 min, followed by 10 cycles including 10 s at 95 °C, annealing at 45–50 °C for 20 s (ramping 0.5 °C/cycle) and 20 s elongation at 72 °C. For the next 25 cycles the annealing temperature was set to 52 °C for 20 s ending with 5 min final elongation at 72 °C (MJ Research PTC-200 Gradient Thermal Cycler, Marshall Scientific, Hampton, New Hampshire, USA).

Outer PCR was performed to attach the barcodes (a sample-specific 10 nucleotide sequence used for de-multiplexing) on the 5' end of the amplicon, which were followed by sequencing adapters. The mixture for outer PCR contained 0.2 μl of DNA polymerase (5 U/μl), 2.5 μl of 10× buffer A (KAPA Taq PCR Kit, Kapa Biosystems, USA) together with 1 μl of 20× EvaGreen™ (Biotium, Fremont, California, USA), 0.5 μl of 10 μM dNTP solution, ~ 20 ng of nucleic acid from the primary PCR and 3 μl of 10 μM primer solution in a total volume of 20 μl. This secondary PCR run was performed for 8 cycles including denaturation at 95 °C for 10 s, annealing at 60 °C for 10 s and 40 s elongation at 65 °C with 3 min final elongation at 72 °C. The quality of the amplification products was visualized by electrophoresis on 1.5% agarose gel after each amplification run. Based on this analysis, some amplicons were discarded due either to an unsuccessful PCR or out of range size.

The size range and quantity of nucleic acid in each individual library were assessed using a DNA 1000 kit on an Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, California, USA) (see Additional file 5). Prior to sequencing, all suitable amplicons were pooled together at equimolar quantities, then purified with E-Gel® SizeSelect™ (Invitrogen, Carlsbad, California, USA) and quantified with a Qubit 2.0 fluorimeter (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Amplicon libraries were concentrated with Omega Cycle Pure Kit (VWR International, Radnor, Pennsylvania, USA) according to the manufacturers' instructions and the subsequent DNA quantification was performed with a Qubit dsDNA Assay Kit (Molecular Probes, Eugene, Oregon, USA) on a Qubit 2.0 fluorimeter (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

Sequencing was carried out on an Ion Torrent PGM™ sequencing platform (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and the library prepared using Ion PGM Hi-Q View OT2 Kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA) according to the manufacturers' instructions. The library was loaded on an Ion 314 chip (Life Technologies, Carlsbad, California, USA), and sequenced for 800 cycles using an Ion PGM Hi-Q View Sequencing Kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

Post-sequencing analysis

Raw sequencing data from PGM runs were imported into CLC Genomics Workbench 10.1.1 (Qiagen, Hilden, Germany) to perform trimming. Briefly, low quality (trimming limit = 0.05) and ambiguous nucleotides, adapters and short residual reads (< 100 nucleotides) were removed. Filtered reads were then assembled into contigs with an overlap-layout-consensus (OLC) approach. The original reads were re-mapped on the assembled contigs to allow visual inspection of the correctness of the assembly of each contig. Sequences supported by less than 3 reads were discarded without further analysis and in some cases contigs displaying a high amount of polymorphism were re-assembled with more stringent parameters to obtain all the possible sequence variants. Transcripts were blasted (using BLASTx) against a custom sequence database containing all known Class-1 (Ranidae and Hylidae) or Class-3 (Bombinatoridae) nucleotide sequences to detect all positive hits based on E-value threshold of 0.05. The longest ORFs for each of the resulting contigs were translated into amino acid sequences using the ExPASy

translate tool [26] and grouped together based on common features.

Peptide characterization

Two peptides with amidated C-terminus were obtained from GenicBio Ltd. (Shanghai, China) at > 98% purity as confirmed by RP-HPLC and MS. They were dissolved in doubly distilled water and stock concentration determined as described previously [27, 28]. Minimal inhibitory concentration (MIC) was determined on a Gram-negative and a Gram positive reference laboratory strains, *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213, obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA), using the serial two-fold microdilution method as described previously [27]. Cytotoxic effects on metabolic activity were determined on human monocytes isolated from buffy coats of informed donors (in accordance with the ethical guidelines and approved from the ethical committee of the University of Trieste) [29], using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay after treatment with the peptides for 24 h, as described previously [30].

Results

Novel peptide sequences

Selective amplification with AMP specific primers of cDNA libraries obtained from seven anuran species (Ranidae, Hylidae, Bombinatoridae families), followed by long-read high-throughput sequencing, resulted in 120.371 reads. Out of the assembled contigs, 5–15% of the sequences from the Ranidae family corresponded to AMPs, depending on the species. The success rate for *Hyla arborea* (1 contig, 0.2%) was much lower (see Table 2). *Bombina variegata* gave no contigs corresponding to AMPs, while *Bombina bombina* amplicons were not pooled with the others for sequencing, due to their excessive average size (573 nucleotides), as evaluated by Agilent Bioanalyzer 2100. This was suggested by the DNA fragment size restrictions (< 450) of the Ion Torrent™ PGM sequencer that was used.

Overall, this approach permitted to identify 128 likely AMP sequences. One hundred twenty seven of these were identified in the five species from the Ranidae family, and only one in *Hyla arborea*, from the Hylidae family (see Table 2). Before proceeding further with the analysis, we extracted the putative mature peptide region by removing the sequence N-terminal to the basic Lys-Arg propeptide cleavage site. The predicted peptides were then checked for identity with known sequences using BLASTp [31] and manually grouped into eight different classes. Seven of these classes were grouped based on the predicted secondary structure (e.g. α-helix, β-sheet), length, frequency of specific amino acids (e.g. presence of a characteristic rana-box domain) [32] and identity with known AMP classes. The eight class consisted of peptides which could not be classified into any of the other seven groups (see Additional file 6). About half of the peptides had already been identified previously in the same, or in closely related, species (48% with 100% identity), or shared significant similarity with previously described AMPs (27% with 81–99% identity). Thirteen of the peptides (10% of the total) showed significant BLAST e-values but < 80% identity with AMPs deposited in the protein sequences databases (see Table 3 and Additional file 7). Additionally, we could identify 16 entirely novel peptides (12%), here defined as peptides lacking significant similarity with known AMPs, but that may have antimicrobial activities based on their physico-chemical characteristics (charge, overall hydrophobicity) and the conserved secretion signal sequence (see Table 3 and Additional file 7). Four additional peptides (3%) with 100% identity to known peptide sequences were also identified. However, with less than 70% query cover and additional amino acids in their primary structure those were also categorized as novel (see Table 3 and Additional file 7). It is worth noting that some of these peptides were found to be identical (at the amino acid level) in multiple analyzed species, thereby reducing the number of completely unique novel peptide sequences to 14, and to 11 for peptides with less than 80% identity. Altogether, these results validate the reliability of this approach, confirming the effectiveness of the method that has been proven to be fast, accurate and suitable for simultaneous identification of

Table 2 Number of raw and trimmed reads, assembled contigs and putative antimicrobial peptides classified per species and the primer of choice

Species	Forward primer	Raw reads	Trimmed reads	Contigs	AMPs (on target)
Pelophylax kl. *esculentus*	TP1	10.278	7.674	399	22 (5%)
	TP2	6.995	3.786		
	TP3	18.067	13.321		
Pelophylax ridibundus	TP1	5.401	4.192	196	31 (15%)
	TP2	3.670	2.446		
	TP3	5.132	3.924		
Rana arvalis	TP1	8.292	6.294	391	31 (8%)
	TP2	12.498	6.272		
	TP3	1.505	1.101		
Rana dalmatina	TP1	4.404	2.697	275	20 (7%)
	TP3	17.884	9.748		
Rana temporaria	TP1	6.751	4.164	187	23 (12%)
Hyla arborea	TP4	11.161	4.690	448	1 (0.2%)
Bombina variegata	TP5	8.333	4.807	577	0

Table 3 Identified putative AMP sequences with no significant identity to the known proteins, less than 80% identity to known AMP sequences or 100% identity to proteins stored at NCBI database with < 70% query cover and their physico-chemical characteristics

#	Sequence	Species	Charge	H[a]
No identity to proteins stored at NCBI nr database				
1	FLGALGNALSRVLGK	*R. temporaria*	+3	0.813
2	FIGALVNALTRVLGK	*R. temporaria*	+3	1.213
3	FIGALVHALTGILGK	*R. temporaria*	+ 2	2.247
4	LVPFIGRTLGGLLARFGK	*R. temporaria*	+ 4	1.500
5	VPQLCFKFQKVIYCEINRTLPNEA	*R. dalmatina*	+ 2	−0.625
6	FSQLFFAWLLRLCRQ	*P.* kl. *esculentus*	+3	2.587
7	GIVEAWPLR	*P. ridibundus*, *R. dalmatina*	+ 1	1.133
8	NNLRHIVAWCKNRNYSLAVCARFKPQ	*R. temporaria*	+ 6	−1.612
9	NLLGFLQGAKDILKECEADNYQGWLCESYKPQ	*R. dalmatina*	−1	−1.250
10	FLPLVLGKTHSEQAEILSWKSSNVEYHLPKCTTDV	*P. ridibundus*, *R. dalmatina*	0	−0.763
11	FLPLIAGLWVNCSANNPKMLKLWK	*P.* kl. *esculentus*	+ 4	1.363
12	FLPICDKSALRFVGKV	*P. ridibundus*	+3	0.494
13	EMPMKKKEETIQKKGMLKWKTIFTSHCWSFE	*P. ridibundus*	+ 4	−1.835
14	RGLLDPITGLVGGLLR	*H. arborea*	+ 2	1.213
< 80% identity to proteins stored at NCBI database				
15	FLGFVGQALNALLGKLGK	*R. dalmatina*	+3	1.550
16	FLPAIAGILSQIFGK	*P.* kl. *esculentus*	+ 2	2.540
17	FFPAFLKVAAKVVPSIICSITKNVET	*P.* kl. *esculentus*	+3	0.573
18	IVPILLGVVPQLVCAITKKC	*R. dalmatina*, *R. temporaria*	+3	1.675
19	IIPLLLGKVVCAITKKC	*R. dalmatina*	+ 4	1.271
20	LVPMFLSKLICFITKKC	*R. temporaria*	+ 4	1.918
21	GLEVLGKILSGILGK	*R. dalmatina*, *Rana arvalis*	+ 2	1.220
22	LLGAALSALSSVIPSVISWFQKG	*Rana arvalis*	+ 2	1.670
23	LANRAARNTSQNVLNAITCTL	*R. dalmatina*	+3	−1.662
24	ADFLDKLRNFAAKNLQNKASL	*P. ridibundus*	+3	−1.595
25	EMLRKKEETIQKKGMLKWKNDFYQSLLEF	*P. ridibundus*	+3	−1.779
100% identity to proteins stored at NCBI database with < 70% query cover				
26	QKTYNRRPPGWSLYVFHQQISNLELEVI	*P.* kl. *esculentus*	+ 2	−0.654
27	FVPLLVSKLVCVVTKNVRIWKLELEII	*Rana arvalis*	+3	1.663
28	FVPLLVSKLVCVVTKNVRTLET	*Rana arvalis*	+3	0.455
29	FLPIVTNLLLRFVG	*R. dalmatina*	+ 2	3.729

[a]Calculated using the CCS consensus hydrophobicity scale [50]

large numbers of AMP sequences from Ranidae. Some potential limitations are discussed in the following section.

Preliminary biological characterization

Two of the novel identified peptides were synthesized (see Table 4) and tested for their in vitro activity against a Gram-negative and a Gram-positive reference bacterial strains. Results are promising, with both peptides active against *S. aureus*, especially rarv_10.1_19 with MIC of 4 μM. On the other hand, peptides don't seem to be selective for Gram-negative strains (see Table 4) with MIC > 64 μM. Another encouraging aspect was the very low toxicity towards human circulating blood cells, namely monocytes. At the highest concentration used (100 μM) over 80% of cells were fully viable (see Table 4).

Discussion

Selective amplicon sequencing resulted in the simultaneous identification of antimicrobial peptide encoding transcripts from 5 out of 7 different anuran species tested. All the species with a positive result pertain to the Ranidae family, with a single sequence obtained from *Hyla arborea* (Hylidae) and none from *Bombina*

Table 4 Peptides tested for biological activity and their physico-chemical characteristics

Peptide[a]	Coding sequence	Sequence[b]	MW	Charge	MIC (μM)[c]		%Viability[d] (100 μM)
					E. coli	*S. aureus*	Monocytes
#4	rtemp_4.3_210	LVPFIGRTLGGLLARF-NH2	1729.1	+ 4	> 64	4	> 80
#22	rarv_10.1_19	LLGAALSALSSVIPSVISWFQK-NH_2	2286.7	+3	> 64	16	> 80

[a]Peptide number refers to designation in Table 3
[b]Sequences end with a C-terminal amidation signal [51, 52]
[c]Evaluated using microdilution assay in MH medium
[d]Viability assessed in the presence of 100 μM peptide after 24 h exposure by the MTT assay

variegata (Bombinatoridae) (see Table 2). The different success rate of this approach among three anuran families can be explained by several factors. First, the assembled transcriptome data used for primer design comprised several species pertaining to either the *Rana* or *Pelophylax* genus (Ranidae), whereas limited data with satisfactory phylogenetic relations was available for Hylidae. The high number of AMP transcripts available in the public databases for the two Ranidae genera enabled to construct three primers suitable for efficiently amplifying the different expressed AMP transcripts in the five target species, improving the chances of correct annealing and amplification in PCR. As the nucleotide sequence data available for other genera increases in the databases, it should become possible to refine primer design and provide suitable primer options also for other more distantly related families.

Another important parameter is transcript size. All known Class-1 Ranidae peptides are shorter than 100 AA long (mostly 70–80). Furthermore, our transcriptome screening revealed that the 3' UTR region of the encoding transcripts was generally shorter than 200 nucleotides. The Ion Torrent™ PGM (Life Technologies, Carlsbad, California, USA) sequencing kit used is suitable for sequencing DNA strands up to about 450 base pairs. The design of degenerate primers based on the Class-1 signal peptide region should therefore have generated amplicons with a size range compatible with this sequencing method. This was confirmed using an Agilent Bioanalyzer 2100 prior to sequencing, which revealed a library size of between 300 and 400 bp for all the Ranidae species (see Additional file 5). This being said, libraries at the top of this size range, at around 400 bp, were very close to the maximal permissible size range, which could create problems. Amplicons successfully obtained from the *Bombina* species were excluded from sequencing for this reason, as their average size was > 500 nucleotides. Using the new isothermal amplification for Ion Torrent, or a different sequencing platform, may partly counteract this issue.

A third consideration comes from a detailed analysis of the data from Ranidae species. We obtained 22 unique assembled sequences in *P.* kl. *esculentus*, 31 in *P. ridibundus*, 31 in *R. arvalis*, 20 in *R. dalmatina* and 23 in *R. temporaria*. These results highlight the considerable sequence diversity of Class-1 AMPs in Ranidae. Identification of such a high number of variants within a single specimen underlines the fact that these AMPs are encoded by multigenic families [3]. Although the high conservation of the signal peptide region allowed targeted DNA sequencing, this feature can represent a potential obstacle in whole transcriptome sequencing. Indeed, the inefficient assembly of full length transcripts, or the collapse of similar variants within a single contig are well-known issues linked to the use of short reads in the assembly of highly similar transcripts derived from multigenic families [33, 34]. Therefore, we suggest the use of longer reads. In this respect, those obtained by Ion Torrent may represent an optimal balance between high-throughput and reasonable length (up to 450 bp) for the management of this sequence diversity. For the same reason, the use of three different primers is another key factor for successful amplification. This guarantees an efficient pairing with all the possible sequence variants. Indeed, even within the Ranidae family, we noticed substantial differences in the efficiency of the amplification using the 3 primers across species (see Additional file 8).

Considering that only one Class-1 AMP encoding transcript was obtained from *H. arborea* (Hylidae family), the explanation could thus be a combination of *i)* the poor representation of transcriptomic datasets from *Hyla* spp. in the SRA database; *ii)* the size range of the library, which was close to the maximum capabilities of Ion Torrent sequencing (see Additional file 5), and *iii)* a different AMP gene organization for this family. With respect to the first consideration, 4 out of 5 transcriptomes used for primer design pertained to a species of a different subfamily (Pelodryadinae) then *Hyla* (Hylinae) and the only transcriptome available for *Hyla arborea* was not obtained from AMP-producing tissues. It thus seems likely the designed forward primer did not include all the polymorphisms present in AMPs from the *Hyla* genus, resulting in the amplification of a single but highly represented sequence (22% of the total sequencing output). Concerning the second consideration, it is likely that AMP amplicons had been removed during the E-gel purification procedure,

due to excessive size. The third consideration could be relevant if, unlike Ranidae family peptides, *Hyla* Class-1 AMPs do not have a multi-gene organization, even though this seems to be disproved by previous reports [3]. Based on these observations a similar approach should be undertaken in the future with an improved primer design, based on broader taxonomical sampling, specifically including other Hylinae species.

Unsuccessful results with Bombinatoridae are most certainly linked to the longer AMP precursor and, consequently, longer length of the encoding mRNA molecules. During the initial phases of the experimental design, we tried to overcome this issue by designing more internal primers based on the propeptide rather than on the signal peptide region, thereby reducing the size of the expected amplicons. However, the assessment of the library size range indicated that amplicons were above (*B. bombina*) or very close (*B. variegata*) to maximal input length capabilities of the sequencing technologies used. While the former library was discarded altogether, the latter one was subjected to sequencing but did not produce any positive matches. Despite a similar apparent size range between the libraries obtained from *B. variegata* and some of the longer ones obtained from Ranidae species, the concentration of the former was approximately 7 times lower (see Additional file 5). The most likely cause of unsuccessful sequencing in this species is therefore the removal of AMP amplicons during the E-gel purification procedure due to their borderline size, similarly to *H. arborea*, so that only non-specific amplicons were sequenced. To confirm this hypothesis we carried out purification of a single 573 bp band of *B. bombina* amplification visible on the agarose gel, followed by sequencing on a Sanger ABI 3130 sequencer (Thermo Fischer Scientific, Waltham, Massachusetts, USA). Although the chromatogram was not clean, suggesting that multiple products of the same size were amplified, the consensus sequence clearly confirmed the amplification of a Class-3 AMP precursor. Therefore, while our strategy was not suitable with Bombinatoridae for Ion Torrent PGM, other massive parallel sequencing platforms allowing higher read lengths (such as SMRT PacBio or Oxford Nanopore) could enable its application also in this anuran family.

Overall, the positive results obtained with Ranidae species, with the identification of 127 peptides, including several novel AMPs (i.e. lacking significant sequence similarity with previously characterized anuran sequences) confirm the effectiveness of this experimental design, as long as the degenerate primers are properly designed, and the amplicon size is tailored to the sequencing platform used. Geographical location would have been very important if the selected species were endemic to Croatia. However, in this case all the species targeted display a relatively broad and partially overlapping area of distribution across Europe and are, in some cases, evolutionarily closely related (e.g. the latest common ancestor of *R. dalmatina*, *R. temporaria* and *R. arvalis* lived in the Miocene [35]). Consequently, the expansion of the taxonomical breadth of sampling to other species adapted to different geographical locations, environmental niches and thereby evolving under different microbial contexts might represent a reliable strategy for novel anuran AMP discovery.

The single result obtained from *H. arborea* indicates that the panel of species analyzed can be quite wide, but this requires a particular effort in collecting as many sequences as possible from species phylogenetically closely related to the target species in the analysis panel to optimize primer design, thereby maximizing the chances of annealing during selective amplification. In this respect, PCR experimental conditions, and the annealing temperature in particular, need to be carefully selected to allow pairing with templates that are not perfectly matching, and allow capturing of as many sequence variants as possible. The lack of success in obtaining Bombinatoridae AMPs instead pinpoints the importance of tailoring the sequencing platform to the expected amplicon length, or alternatively, to identify useable sequences and design primers as close as possible to 3′ end of the mRNAs in order to reduce the amplicon size.

Although the novel identified peptides display physico-chemical features compatible with antimicrobial activity (see Table 3) and clearly possess a well-conserved signal peptide/propeptide region typically found in anuran AMPs, their biological role requires confirmation. A comprehensive evaluation of the antimicrobial activity of six selected novel peptides identified in this study is currently in progress, and preliminary results are presented for two of these peptides (see Table 4). This confirms the reliability of our approach to identify novel, functional antimicrobial peptide sequences (manuscript in preparation).

Conclusions

The approach here presented, with suitable modifications, can be applied also to other gene families sharing a conserved signal peptide and/or propeptide region, an hypervariable mature peptide region, and a limited distance between this region and the mRNA poly(A) tail. These characteristics are well known in many different animal AMPs [36–41], toxins [42–44] and other types of bioactive peptides from other organisms [45]. One should however always keep in mind a key factor, i.e. the detection of sequence variants depends on their being expressed. Therefore, whenever possible, the most appropriate tissue and/or

experimental challenge need to be selected to enhance the expression of the target mRNAs. In our case, the choice of anuran skin was amply supported by abundant literature [5, 46–49], and indeed we could obtain several dozens of different peptides for each species as expected. However, the number of reads obtained for each sequence variant does not necessarily depend only on the level of expression of the transcript itself but is also affected by the efficiency of the amplification, which depends on the match to the primer. For this reason, the results of this type of study can only be considered as qualitative, and not as a proxy to investigate the expression levels of AMP variants. Finally, the small amount of tissue required may permit the identification of novel AMPs from endangered species, potentially without the need for sacrificing any individual. This would permit to fully exploit animal biodiversity in identification of potential novel therapeutic agents, without adding to the threat of reducing it.

Additional files

Additional file 1: Complete list of frog species obtained from Croatian wild. (DOCX 13 kb)

Additional file 2: List of transcriptome data downloaded from SRA database. (DOCX 14 kb)

Additional file 3: List of signal peptides used for transcriptome screening. (XLSX 46 kb)

Additional file 4: Clusters of nucleotide alignments used for forward primer design. (DOCX 7338 kb)

Additional file 5: Size range of each individual library obtained prior to pooling. (DOCX 14 kb)

Additional file 6: Classification of identified peptides. (DOCX 1600 kb)

Additional file 7: BLASTp output for novel identified putative AMP sequences. (XLSX 47 kb)

Additional file 8: Success rate of amplicon synthesis in Ranidae species based on used primer. (DOCX 13 kb)

Abbreviations

AMP: Antimicrobial peptide; OLC: Overlap layout consensus; ORF: Open reading frame; RACE: Rapid amplification of cDNA ends

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Availability of data and materials

The datasets generated and/or analyzed during the current study are available in the NCBI SRA database under BioProject accession ID: PRJNA415374.

Authors' contributions

TR performed transcriptome screening, primer design, post-sequencing bioinformatic analysis, RNA extraction, cDNA amplification, assisted with the library preparation and wrote the paper, MG performed assembly, post-sequencing bioinformatic analysis, assisted with transcriptome screening and primer design and wrote the paper, FS performed cDNA amplification and library preparation, FF performed library preparation and sequencing, SM collected biological material, AT helped conceive and design the experiments and contributed to post-sequencing peptide analyses, AP conceived and designed the experiments and contributed to post-sequencing nucleic acid bioinformatic analyses. All authors provided a critical contribution for the preparation of the manuscript and approved its final version.

Ethics approval and consent to participate

The Ethics Committee at the Faculty of Science, University of Split, approved the use of Croatian frog species for the purpose of this research.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Publisher's Note

Author details

[1]Department of Physics, Faculty of Science, University of Split, 21000 Split, Croatia. [2]Department of Life Sciences, University of Trieste, 34127 Trieste, Italy. [3]Public Institution for the Management of Protected Areas in the County of Split and Dalmatia – "Sea and karst", 21000 Split, Croatia.

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2 Membrane-active antimicrobial peptide identified in *Rana arvalis* by targeted DNA sequencing

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In a follow-up research to the first paper, some of the twenty nine novel identified peptide sequences were functionally characterized. Considering the high manufacturing costs, experiments involving all the identified peptides would have been too expensive and time-consuming. For this reason, a filtering process was deemed to be advisable. There are devoted algorithms, freely available online, which can assist in such a decision, which however can sometimes be misleading. The six peptides chosen for chemical synthesis and activity verification were selected considering molecular diversity to cover a wide range in physico-chemical properties. From this pool, one peptide stood out in terms of antibacterial activity, especially against *Staphylococcus aureus*. Other peptides showed moderate to no antibacterial activity. All six peptides proved to be non-toxic to human cells with the most potent, Peptide-6, showing some toxicity against one cell line, but at a range of concentrations well above its antibacterial concentration. Interestingly, the activity of this peptide seemed to correlate with its ability to self-associate/oligomerize in the presence of the membrane, as deduced using circular dichroism and surface plasmon resonance experiments with liposomes. This feature is rather interesting and could be one of future directions for AMP research in our lab when it comes to potent peptides with possible biomedical applications. Resistance towards antibiotics is a constantly growing problem (see Introduction), encouraging the continued validation of the most successful candidates in pre-clinical trials.

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Membrane-active antimicrobial peptide identified in *Rana arvalis* by targeted DNA sequencing

Tomislav Rončević[a,*], Lucija Krce[a], Marco Gerdol[b], Sabrina Pacor[b], Monica Benincasa[b], Filomena Guida[b], Ivica Aviani[a], Vedrana Čikeš-Čulić[c], Alberto Pallavicini[b], Ana Maravić[d], Alessandro Tossi[b]

[a] Department of Physics, Faculty of Science, University of Split, Croatia
[b] Department of Life Sciences, University of Trieste, Italy
[c] Department of Medical Chemistry and Biochemistry, School of Medicine, University of Split, Croatia
[d] Department of Biology, Faculty of Science, University of Split, Croatia

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ABSTRACT

Antimicrobial peptides (AMPs) are naturally produced, gene encoded molecules with a direct antimicrobial activity against pathogens, often also showing other immune-related properties. Anuran skin secretions are rich in bioactive peptides, including AMPs, and we have reported a novel targeted sequencing approach to identify novel AMPs simultaneously in different frog species, from small quantities of skin tissue. Over a hundred full-length peptides were identified from specimens belonging to five different Ranidae frog species, out of which 29 were novel sequences. Six of these were selected for synthesis and testing against a panel of Gram-negative and Gram-positive bacteria. One peptide, identified in *Rana arvalis*, proved to be a potent and broad-spectrum antimicrobial, active against ATCC bacterial strains and a multi-drug resistant clinical isolate. CD spectroscopy suggests it has a helical conformation, while surface plasmon resonance (SPR) that it may self-aggregate/oligomerize at the membrane surface. It was found to disrupt the bacterial membrane at sub-MIC, MIC and above-MIC concentrations, as observed by flow cytometry and/or visualized by atomic force microscopy (AFM). Only a limited toxicity was observed towards peripheral blood mononuclear cells (PBMC) with a more pronounced effect observed against the MEC-1 cell line.

1. Introduction

AMPs or host defense peptides (HDPs) are structurally diverse multifunctional effector molecules naturally produced by all organisms having a direct antimicrobial activity and/or immunomodulatory properties [1,2]. They are considered to be potential new therapeutic agents useful in a battle against the rapidly spreading multi-drug resistant bacteria [3], with many peptides in phase I–III of clinical phase of development [4,5]. However, there are a number of limitations in development of potential peptide antibiotics, resulting in only a handful of AMPs approved for clinical use to date. Some major concerns are low metabolic stability and short half-life (lability to proteases, serum, salt, pH), a tendency for aggregation, unacceptable toxicity and high production costs [4–6]. Nevertheless, AMPs are often membrane-active antibiotics, with a mechanism that is to a certain extent similar to that of colistin, which in recent years has been used as a “drug of last resort” against Gram-negative pathogens. However, its effectiveness could also become hindered due to reported cases of resistance [7], especially by plasmid-mediated dissemination of the *mcr-1* gene [8].

AMPs are an abundant source of novel potential antibiotics, and according to Data Repository of Antimicrobial Peptides (DRAMP) [9], 4851 AMPs with diverse structure and activity have been reported. Anurans, and in particular their skin secretions, are a rich source of such peptides [10]. Since the first frog peptide was identified from the

Abbreviations: DiOC6, 3,3′-dihexyloxacarbocyanine iodide; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DPPG, 1,2-dipalmitoyl-*sn*-phosphatidylglycerol; AFM, atomic force microscopy; AMPs, antimicrobial peptides; CD, circular dichroism; LUVs, large unilamellar vesicles; ORFs, open reading frames; SPB, sodium phosphate buffer; SDS, sodium dodecyl sulphate; SPR, surface plasmon resonance; TFA, trifluoroacetic acid; TFE, trifluoroethanol

* Corresponding author.
E-mail addresses: troncevic@pmfst.hr (T. Rončević), lkrce@pmfst.hr (L. Krce), mgerdol@units.it (M. Gerdol), pacorsab@units.it (S. Pacor), mbenincasa@units.it (M. Benincasa), milenaguida@gmail.com (F. Guida), iaviani@pmfst.hr (I. Aviani), vedrana.cikes.culic@mefst.hr (V. Čikeš-Čulić), pallavic@units.it (A. Pallavicini), amaravic@pmfst.hr (A. Maravić), atossi@units.it (A. Tossi).

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skin secretions of *Xenopus laevis* in the late 1980s [11], numerous AMPs have been identified in most of anuran families (e.g. 1102 peptides deposited in DRAMP have been identified in frogs) and in particular in Ranidae [12]. Frogs from this family are known to secrete multiple active AMPs [12], which are likely expressed by relatively large gene families, as also confirmed by a study we recently carried out on five different species [13]. In the past, identifying novel anuran AMPs required handling a number of individuals which were stimulated by noradrenaline (e.g. norepinephrine) to obtain small amounts of crude peptide. Following initial fractionation by RP-HPLC, potential peptides were identified by activity testing of the obtained fractions. Active components were then purified, and the sequence determined by different methods, including mass spectrometry [14]. Although effective, this approach is very time consuming, and more importantly raises issues related to the ethical treatment of animals and to the protection of amphibian species which are often experiencing a sharp population decline due to habitat destruction and anthropic activities [15].

We have previously reported an alternative, high-throughput method for AMP identification which is faster, more efficient, less invasive and allows for simultaneous peptide identification from several individuals belonging to different species [13]. Implementing degenerate primers designed on the most conserved regions of AMP precursors, the signal sequence, allowed us to simultaneously identify 127 peptide sequences from five different Ranidae species, including 29 novel sequences. Six peptides were then selected for synthesis and tested for their antibacterial potential against a panel of Gram-negative and Gram-positive bacteria, including methicillin-resistant *Staphylococcus aureus*. The toxicity towards animal cells was examined on circulating human blood cells and the MEC-1 cell line. The peptides showed a quite variable antimicrobial activity, ranging from broad spectrum to selective or completely inactive, and antimicrobially active peptides were relatively nontoxic towards animal cells in vitro, at concentrations corresponding to their MIC or MBC. The most active peptides were examined for their ability to bind with the surface of model biological membranes and to perturbate or disrupt bacterial membranes. Based on surface plasmon resonance (SPR) results and CD spectra, it was possible to correlate the potency of the most active peptide to its ability to self-aggregate/oligomerize on interaction with the model or bacterial membranes, which could have also contributed to a moderate toxicity towards some types of host cells.

2. Materials and methods

2.1. Peptide identification and relatedness

Peptides were identified from different Ranidae species including *Pelophylax ridibundus*, *Pelophylax* kl. *esculentus*, *Rana dalmatina*, *Rana temporaria* and *Rana arvalis*, as described in detail in Roncevic et al. [13]. Briefly, complete RNA was extracted from ~50 mg of skin tissue. Simultaneously, signal peptide sequences belonging to Ranidae species were obtained from Database of Anuran Defense Peptides (DADP) [16], aligned and used to generate Hidden Markov Model (HMM) profiles. The RNAseq data available in the Sequence Read Archive (SRA) database [17] for Ranidae was then probed by using these HMM profiles and *de novo* assembled, enabling the identification of expressed sequences encoding for potential AMPs. Positively hit nucleotide sequences were aligned and grouped by similarity (> 80%) in three clusters, which allowed for the design of degenerate forward primers potentially able to target the entire molecular diversity of Ranidae AMPs. The reverse primer matched the poly-A tail of the mRNA. Amplicons resulting from cDNA PCR amplification were size selected and sequencing was carried out on an Ion PGM™ sequencing platform (Thermo Fisher Scientific, USA). Upon standard trimming procedure, reads were assembled into contigs with an overlap-layout-consensus (OLC) approach. Finally, AMP-encoding sequences were identified by similarity and virtually translated using the ExPASy translate tool [18].

Relatedness of the identified AMPs among themselves and with previously described anuran AMPs was carried out by a combination of BLASTn and multiple sequence alignment, principally concerning the nucleotide sequences of the propeptide and 3′UTR of the encoding mRNAs. These regions are relatively well conserved in AMPs and the identity can therefore be used to infer relatedness.

2.2. Peptide synthesis

Selected frog peptides were obtained from GenicBio Limited (Shanghai, China), all at > 98% purity and amidated at C-terminus, except Peptide-3 and -4, which had an acidic C-terminus and one disulphide bridge each. The correct structures were confirmed by RP-HPLC/MS (see Fig. S1). Chromatographic separation was achieved on a reversed-phase column (C18, 5 µm, 110 Å, 4.6 × 250 mm) using a 25–85% 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 [19].

2.3. Preparation of liposomes

LUVs (large unilamellar vesicles) were prepared by dissolving dry lipids, 1,2-dipalmitoyl-*sn*-phosphatidylglycerol (DPPG) and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) (Avanti Polar Lipids, Alabaster, Alabama, USA) in chloroform/methanol (2:1) solution. The solution was evaporated using a dry nitrogen stream and vacuum-dried for 24 h. The dry lipid cake was resuspended in 1 mL of sodium phosphate buffer (SPB) 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 before passing through a mini-extruder (Avanti Polar Lipids) successively using polycarbonate filters with 1 µm, 0.4 µm and 0.1 µm pores. The vesicles were resuspended to a final phospholipid concentration of 0.4 mM and 1 mM, for circular dichroism (CD) and surface plasmon resonance (SPR) experiments, respectively. Based on the bilayer membrane surface area of a ~100 nm diameter liposome, and the area of a phospholipid head group (~0.7–1 nm^2) [20] the concentration of liposomes is estimated at between 5 and 10 nM.

2.4. Circular dichroism

CD spectra were obtained on a J-710 spectropolarimeter (Jasco, Tokyo, Japan). The spectra are accumulations of three scans measured in *a*) SPB solution, *b*) 50% TFE in SPB, *c*) the presence of sodium dodecyl sulphate 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 per/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. [21].

2.5. Antibacterial activity

Minimal inhibitory concentration (MIC) was determined on four Gram-negative and Gram-positive laboratory strains from the American Type Culture Collection (ATCC, Rockville, MD, USA) including *E. coli* ATCC 25922, *Acinetobacter baumannii* ATCC 19606, *Staphylococcus aureus* ATCC 29213 and *Enterococcus faecalis* ATCC 29212 and multidrug resistant *S. aureus*. Its origin, antibiograms, and characterization of resistance determinants were described previously in Roncevic et al. [22]. Results were obtained using the serial two-fold microdilution method according to CLSI [23]. Bacteria were cultured in a fresh Mueller Hinton broth (MHB) (Biolife, Milano, Italy) or Enterococcus broth (BN Biosciences, Franklin Lakes, New Jersey, United States) to the

mid exponential phase, added to serial dilutions of synthetic 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 or enterococci 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. Surface plasmon resonance

Interaction studies between selected peptides and model membranes were carried out on X100 instrument (Biacore, GE Life Sciences, Chicago, Illinois, United States) immobilizing integral liposomes on an L1 sensor chip surface [24]. DOPC liposomes were injected three times for 10 min with flow rate of 5 μL/min over the L1 sensor surface, until 9000 RU maximum was reached [25,26]. The binding of the peptides with the liposomes was determined injecting a peptide concentration series (0.5 to 32 μM) at 10 μL/min for a contact time of 540 s, followed by a dissociation time of 1200 s with PBS running buffer. Sensorgrams were obtained using BIAevaluation software v 1.1 (Biacore, GE Life Sciences) fitting the curves with the "Affinity - Steady State" mathematical model and then elaborated using GraphPad v 6.04 (GraphPad Software, La Jolla, California, USA). Each experiment was repeated two times.

2.7. Membrane integrity assay

The effect of the peptide-6 on bacterial membrane integrity was studied by measuring the percentage of propidium iodide (PI) positive cells after exposure to the peptide, using an Accuri C6 flow cytometer (BD Biosciences, CA, USA). Measurements were carried out on *S. aureus* ATCC 29213 cells which were cultured in MH medium to the mid logarithmic phase. After incubation, SYTO9/PI dye (LIVE/DEAD® BacLight™ Bacterial Viability Kits, Molecular Probes, Eugene, Oregon, USA) was added to the bacterial suspension (1×10^6 CFU/mL). Dye mixture was prepared and added to the suspension according to the manufacturer's instructions. Peptide was then added in concentrations corresponding to MIC, ½ MIC and ¼ MIC, just before the beginning of the analysis, and the measurement taken after 15, 30 and 60 min. Melittin was used as positive control, while stained untreated cells in MH medium were used as negative control. Non-stained cells and single stained samples were used to compensate fluorescence channels on the cytometer and adjust appropriate gates on dot-plots. Each measurement was conducted in triplicate, and for each incubation time at least 10,000 cells were collected. Data analysis was carried out with FlowLogic 6.0 software.

2.8. AFM images

S. aureus ATCC 29213 cells were investigated by AFM. Over-night culture growth, next-day dilutions and treatments were carried out as reported in Roncevic et al. [27]. Briefly, the bacteria were treated with 2 μM, 4 μM (corresponding to MIC and MBC) and 8 μM of the peptide-6 and incubated with shaking at 100 rpm at 37 °C for 1 h. The culture was then briefly centrifuged, and the pellet resuspended in one tenth of the supernatant. Melittin treated cells were prepared in the same way and treated with the peptide at 2 μM and 3.5 μM, corresponding to MIC and MBC respectively [28]. The control samples were prepared in the same fashion but without the peptide treatment. Bacterial adhesion to glass slides was enabled with Cell-Tak solution (Corning, NY, USA) coating [29] as reported in [27]. AFM measurements were carried out in contact mode, under ambient conditions, using Bruker Multimode 3 (Digital Instruments, USA) instrument with a 0.12 N/m silicon-nitride probe (Bruker AFM probes USA, DNPS-10). Scan rates during imaging were kept between 2 and 3 Hz, and the image resolution was 512 pixels per line. Analysis of the obtained images was carried out with Gwyddion.

2.9. Toxicity assay

The cytotoxic effect of peptides was determined initially on a human MEC-1 lymphoid tumor cell line [30]. Cells were cultured in RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% foetal bovine serum (FBS) (complete medium) and sub-cultured two–three times a week for maximum of 20 passages. Cells were counted by Trypan Blue exclusion test, diluted to 10^6 cells/mL and plated into 96-well culture plates. Cytotoxicity assays were also carried out on human monocytes and lymphocytes isolated from buffy coats of informed donors (in accordance with the ethical guidelines and approved from the ethical committee of the University of Trieste) [31]. Briefly, the buffy coats were diluted 1:1 with PBS and added to an equal volume of Histopaque-1077. After centrifugation for 30 min at 400 × *g*, the white band at the interphase between the plasma and the Histopaque fractions was recovered, transferred into a sterile tube and washed twice with PBS. The cell pellet was then resuspended in RPMI HEPES and transferred to a cell culture flask or multiwell plates. Monocytes were left to adhere for 90 min and then lymphocytes were recovered, as cells in suspension that were used within two days, maintaining them in RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin and 10% foetal bovine serum (FBS) added with HEPES (25 mM), nEAA (1 mM), sodium pyruvate (1 mM), and 2ME (0.05 mM). Adherent monocytes, after gentle washing with warmed PBS, were cultured in complete medium and used within two days. The cells were incubated in humidified air with 5% CO_2 at 37 °C.

Metabolic activity was determined after treatment with the peptides in complete medium for 24 h. For this purpose, 20 μL of MTT dye (5 mg/mL) was added to each well (10^5 cells/well) and incubated for 4 h at 37 °C. Formazan crystals were solubilized with acidic isopropanol (0.04 N HCl in absolute isopropanol) and the absorbance measured at 540 nm and 630 nm with microplate reader (Tecan Sunrise, Männedorf, Switzerland). All measurements were done at least in triplicate.

In order to discriminate viable, late apoptotic and/or necrotic cells, flow cytometry measurements have been carried out on a Cytomics FC500 instrument (Beckman Coulter Inc., Fullerton, CA, USA), equipped with an argon laser (488 nm, 5 mV) and standard configuration. Prior to peptide treatments, cells were double stained with fluorescent probes, namely DiOC6 (3,3′-dihexyloxacarbocyanine iodide) (FluoProbes, Interchim, Montlucon Cedex, France) and PI. Briefly, DiOC6 was used as a marker of mitochondrial functionality and was incubated with the cells for 15 min at 37 °C (final concentration of 50 nM), washed twice with 2 mL of PBS and cells resuspended in PBS. Membrane integrity was assessed with PI which was added to DiOC6-stained cells at final concentration of 15 μM. Then, the cells were treated with peptides up to 60 min and measurement taken every 15 min. Cells treated with 50 μM of the uncoupler carbonyl cyanide 3-chlorophenylhydrazone (CCCP) at 37 °C for 15 min were run in parallel as positive control (collapse of mitochondrial transmembrane potential). Data analysis was performed with the FCS Express3 software (De Novo Software, Los Angeles, CA, USA). Data obtained from repeated experiments were subjected to computer-assisted analysis using GraphPad Instat 3, and statistical significance was assumed at $p \leq 0.05$ (ANOVA, Student-Newman-Keuls post-test).

Table 1
Ranidae peptide sequences and their physico-chemical characteristics.

Peptide	Coding sequence[a] [13]	Sequence[b]	Charge	H[c]
Peptide-1	rtemp_4.3_210	LVPFIGRTLGGLLARF-NH_2	+4	2.5
Peptide-2	rtemp_4.6_20	FLGALGNALSRVL-NH_2	+3	1.9
Peptide-3	rtemp_32_47	NNLRHIVAWCKNRNYSLAVCARFKPQ	+6	−1.6
Peptide-4	rdal_39_24	NLLGFLQGAKDILKECEADNYQGWLCESYKPQ	−1	−1.3
Peptide-5	rdal_8_600	FLGFVGQALNALLGKL-NH_2	+3	2.5
Peptide-6	rarv_10.1_19	LLGAALSALSSVIPSVISWFQK-NH_2	+3	1.9

[a] Peptide name consists of the first letter of the frog genus where the peptide was identified, followed by 3 or 4 letters pertaining to the species name. First number refers to the contig where the peptide was found, and the second one to the number of reads supporting it.
[b] All amidated sequences end with a C-terminal amidation signal (-GK, -GKR) [34,35].
[c] Calculated using the CCS consensus hydrophobicity scale [33].

3. Results and discussion

3.1. Novel peptide sequences

Selective amplification with AMP specific primers of cDNA libraries obtained from five Ranidae species allowed for high-throughput, parallel identification of 127 peptides. Out of those, 29 sequences had either no identity, < 80% identity or 100% identity but with < 70% query cover, with known protein sequences when searched by BLAST [32] against the non-redundant protein database (described in detail in Roncevic et al. [13]). Six peptides were selected for chemical synthesis and activity verification. These peptides were selected on the basis of their different charge (from −1 to +6), hydrophobicity (from −1.6 to 2.5 calculated using the CCS consensus hydrophobicity scale [33]), structural aspects (likely helical conformation or presence of a disulphide bridge) and/or amidated C-terminus (see Table 1). Peptide-3 and -4 contain two Cys residues which likely result in the formation of a disulphide bridge. All other peptides are linear and most likely adopt a helical conformation to some extent.

3.2. Comparison to previously characterized anuran AMPs

As previously reported [13], the six peptides selected for synthesis were chosen based on both molecular diversity and sequence novelty, i.e. the lack of detectable similarity of the mature peptide region with previously characterized anuran AMPs. However, the detailed investigation of the propeptide and 3′UTR regions of the encoding mRNAs might provide meaningful information concerning the evolutionary origins of these sequences. Indeed, both regions are expected to be subject to purifying selection due to their regulatory roles in AMP processing/maturation and post-translational regulation [36–38].

From an evolutionary perspective, peptide-1 and -2 from *R. temporaria* appear to be closely related to previously characterized temporins, including temporin-A (rtemp_1.10_80), -B (rtemp_1_852), -C (rtemp_2_141) -F (rtemp_107_11), -L (rtemp_4.21_24), (the codes indicate that these known sequences were among the ones revealed by our DNA sampling method, see Roncevic et al. [13]), as well as to the novel AMP sequence rtemp_4.7_33. Two of the newly synthesized peptides (peptides-1 and -2, corresponding to rtemp_4.3_210 and rtemp_4.6_20) share significant homology with known temporins, in particular in the propeptide region (100% and 92.78% identity with temporin-A, respectively), and in the 3′UTR regions (96.70% and 86.32% identity with temporin-F and -L, respectively) (see Table S1). Moreover, the length of the mature peptide region of rtemp_4.6_20, including the C-terminal –GK amidation signal, is identical to that of all other temporins (15 amino acids), whereas the slightly superior length of rtemp_4.3_210 can be linked to the presence of a 9 residue insertion within the ORF. Altogether, these data suggest a relatively recent origin of the two novel peptides from the duplication of a temporin gene, followed by rapid diversification of the mature peptide region by positive selection.

On the other hand, while peptide-3 from the same species (rtemp_32_47) does not conform to any cluster of paralogous sequences, it shows significant similarity with rdal_39_24 from *R. dalmatina*, another of the peptides selected for synthesis (peptide-4). Indeed, the two sequences score reciprocal best BLAST hits in inter-species comparisons, due to the presence of highly conserved sequence stretches in the propeptide (77 identical nucleotides out of 80) and 3′UTR (46 identical nucleotides out of 50) regions, which suggest that the two sequences are orthologous. In spite of the presence of multiple indels within the ORF in the mature peptide region, both sequences are characterized by the presence of two cysteine residues and by an unusual KPQ C-terminal motif. Curiously, no other potential orthologous (nor paralogous) sequence could be identified in any of the other target anuran species, including the congeneric *R. arvalis*.

The fifth peptide selected for de novo synthesis, rdal_8_600 from *R. dalmatina*, also appears to be related to temporins, due to a high level of identity with rdal_21_605 (temporin-F) and rdal_40_14 (also belonging to the temporin sequence cluster). Compared to these two sequences, rdal_8_600 displays a 9 nucleotide long insertion within the mature peptide region, maintains the C-terminal –GK amidation signal and shows a 93.5% identical 3′UTR. No clear orthologous AMP sequence could be detected in any of the other target Ranidae species.

Peptide-6 (rarv_10.1_19) from *R. arvalis*, does not conform to any known AMP family and, at the same time, appears to be present as a single-copy gene in all *Rana* species. Sequences clearly orthologous to peptide-6 could in fact be retrieved with our approach in *R. temporaria* (rtemp_88_19 – FIGSALKVLAGVLPSVISWVKQG, processed sequence - FIGSALKVLAGVLPSVISWVKQ-NH_2) and *R. dalmatina* (partial sequence, possibly due to a poor expression level). Rtemp_88_19 was initially discarded [13] discarded due to a poor support by sequencing data. It shares the same length and 74% sequence identity with peptide-6 at the amino acid level.

3.3. Peptide structure

The CD spectra of all six peptides were measured under different conditions, including aqueous buffer, anionic SDS micelles, 50% trifluoroethanol and anionic (DPPG) or neutral zwitterionic (DOPC) LUVs. All peptides are substantially disordered in an aqueous environment, although peptides-3 and -4 show some structuring, likely due to the presence of a disulphide bridge (see Fig. 1). Peptide-1 showed a marked conformational change in the presence of TFE to a spectrum typical for an α-helix. The transition is less marked in the presence of SDS or of neutral LUVs, but quite as marked in the presence of anionic LUVs, although the shape is different (see Fig. 1 and Table S2). We have previously explained the dominance of a minimum at 225 nm with respect to 208 as being due either to self-aggregation at the membrane surface or to the adoption of a 3–10 type helical conformation [27]. In any case it suggests a selective interaction with anionic membrane surfaces. Peptide-2 instead behaves as a canonical helical peptide under all conditions except aqueous, and again the effect is strongest in the

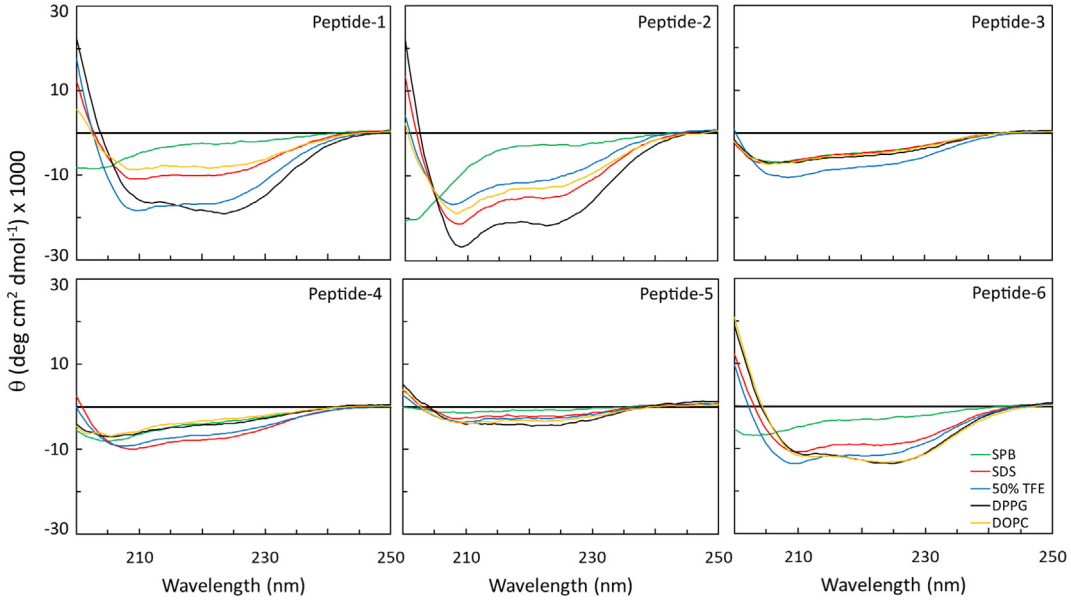

Fig. 1. CD spectra of frog peptides under different conditions. Spectra are the accumulation of three scans carried out with 20 μM peptide in SPB, 10 mM SDS in SPB, 50% TFE, anionic LUVs in SPB (DPPG, 5 nM) and neutral LUVs in SPB (DOPC, 5 nM).

presence of DOPC LUVS, suggesting it inserts into these membranes as a lone α-helix (see Fig. 1 and Table S2). Peptides-3 and -4 show the least change in conformation, which may indicate that they do not interact strongly with a membrane like environment, or that the interaction does not markedly affect their conformation. Note that while a lack of interaction would not be surprising for peptide-4 with DPPG LUVs, as it is anionic, for peptide-3 which is strongly cationic interaction with the surface of DPPG LUVs is to be expected. Peptide-5 shows rather weak spectra but it substantially follows the same trend as peptide-1. Peptide-6 instead differentiates from peptide-1 in that the spectra are very similar in the presence of DPPG or DOPC LUVs, again showing a minimum at 225 nm (see Fig. 1 and Table S2). This would suggest that peptide-1 could be more selective of bacterial membranes with respect to host cell membranes than peptide-6.

3.4. Antibacterial activity

The antibacterial activity of Ranidae peptides was assessed against four reference bacterial strains and one clinical isolate. Out of the six tested peptides, only peptide-6 showed appreciable antibacterial potential (see Table 2) with a certain selectivity towards Gram-positive bacteria, especially *S. aureus* (MIC = MBC = 4 μM). The activity was also tested against a multi-drug resistant isolate of *S. aureus*, with only moderately increased MIC and MBC (8 and 16 μM, respectively). Peptide-6 also showed a moderate activity against *E. faecalis* (MIC and MBC = 16 μM). Out of the remaining five peptides, peptide-1, -2 and -5 showed some activity against *S. aureus* with MIC values ranging from 16 to 32 μM (see Table 2). It should however be noted that when tested in reduced medium (20% MH), MIC values decreased several-fold for all the above peptides (see Table S3) suggesting that antimicrobial activity is rather medium/salt sensitive. This is, in fact, rather common for antimicrobial peptides and is one of the reasons why clinical trials are mostly limited to topical applications (e.g. pexiganan for treatment of impetigo and diabetic ulcers) [5]. Peptide-3 and -4 were inactive, irrespective of the medium conditions used, so they may not be conventional AMPs but have other biological functions [39], or the bacterial susceptibility to these AMPs may be significantly higher in the particular ionic environment in the physical district where they are produced, analogously to previously reported examples for mammalian AMPs [40].

3.5. Model membrane interaction

Interaction with the DOPC membrane was evaluated on peptide-1 and -6. The binding sensorgrams indicate that both peptides are able to bind a model membrane, since the binding response units (RU) increased with the peptide concentrations (see Fig. 2), but the interaction seems to be somewhat different. During the dissociation phase, the binding curve for peptide-1, at 32 μM, does not return to the baseline after washing with PBS, indicating the formation of stable and irreversible complex with the membrane (see Fig. 2 a)). The binding curve does not reach saturation binding so the K_D value was only approximately estimated to be 5×10^{-4} M (see Fig. 2 b)). For peptide-6, binding increased sharply and non-linearly with concentration, with a significant jump in the signal at 16 μM, suggesting self-aggregation and/or oligomerization on the liposome surface [41] (see Fig. 2 c) and

Table 2
Antibacterial activity (μM) of Ranidae peptides against Gram-negative and Gram-positive bacterial strains in MH medium.

Bacterial strains	Peptide-1		Peptide-2		Peptide-3		Peptide-4		Peptide-5		Peptide-6	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
S. aureus ATCC 29213	16	16	32	64	> 64	/	> 64		32	32	4	4
S. aureus c.i.	64	64	NA[a]	NA	NA	NA	NA	NA	NA	NA	8	16
E. faecalis ATCC 29212	> 64	/	> 64	/	> 64	/	NA		> 64	/	16	16
E. coli ATCC 25922	> 64	/	> 64	> 64	> 64	/	> 64		> 64	/	> 64	/
A. baumannii ATCC 19606	> 64	/	> 64	/	> 64	/	NA		> 64	/	32	64

[a] NA = not available.

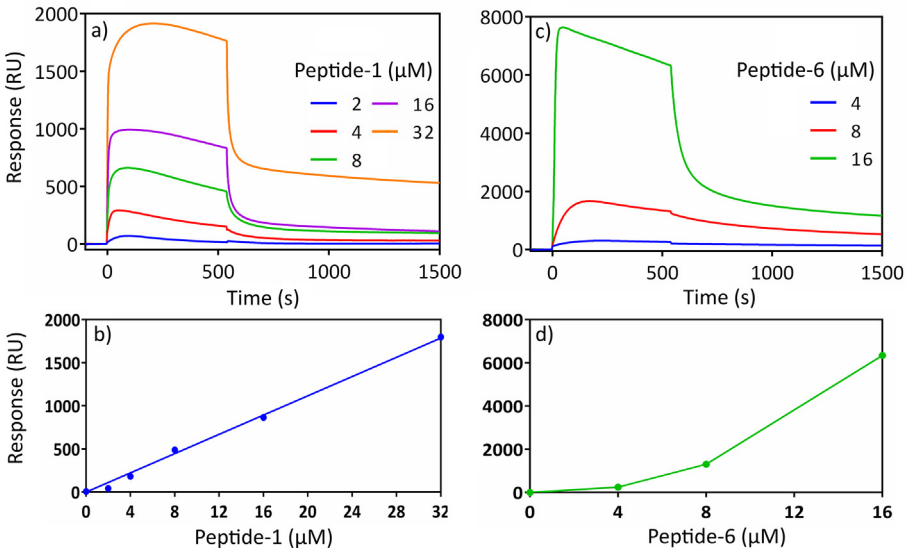

Fig. 2. Binding sensorgrams (top) and binding curves (bottom) for peptide-1 (A–B) and peptide-6 (C–D). Sensorgrams were obtained by applying peptides at increasing concentrations over DOPC LUVs immobilized on a L1 sensor chip. Binding curves were fitted using the "Affinity-Steady State" mathematical model. Shown is one experiment out of two different evaluations with very similar results.

d)). These effects make it difficult to estimate a proper "binding curve" for peptide-6 so that it was not possible to calculate the K_D value.

These results are in agreement with CD spectra, where self-aggregation/oligomerization is indicated by the $\theta^{208}/\theta^{222}$ ratio and were observed for peptide-6 in the presence of both DPPG model membrane (mimicking the anionic bacterial membrane), and DOPC LUVs (mimicking neutral host cell membrane). Interaction and self-aggregation/oligomerization in the presence of the different types of membranes may be related to antimicrobial (see Table 2) and cytotoxic effects on host cells (see below).

3.6. Effect on bacterial membrane integrity

Peptide-6 was further evaluated for its effect on the *S. aureus* membrane integrity, as the peptide was most active against this particular strain (see Table 2). AFM imaging was used to visualize cell wall damage, while flow cytometry was used to monitor PI uptake. Significant PI uptake is often observed for other α-helical peptides, that tend to be membranolytic [42]. Treatment of bacterial cells with peptide-6 caused only slight membrane permeability at sub-MIC concentrations, with < 20% PI+ cells after 1 h incubation (see Fig. 3). The effect is somewhat more pronounced at MIC (4 μM), and is time dependent, with PI+ cells increasing to ~40% after 60 min exposure (see Fig. 3).

The effect of peptide-6 on *S. aureus* cell wall was also investigated by AFM, as compared to untreated cells. The latter showed a smooth cell wall surface without visible perturbations or disruptions. Cells have a characteristic cocci shape and remain attached post division (see Fig. 4 a)). After treatment with peptide-6 at sub-MIC concentration (2 μM), the cell morphology did not change to a significant extent (see Fig. 4 d)), while treatment at 4 μM resulted in significant cellular damage for some of the cells while some remained relatively undisturbed (see Fig. 4 e)). This effect is mostly pronounced at 2 × MIC (8 μM) as all cells are ruptured and their morphology is altered (see Fig. 4 f)). It is interesting to note that cell wall damage is less evident for the cells treated with the known membranolytic peptide melittin at its MIC (2 μM) and MBC (3.5 μM) (see Fig. 4 b) and c)). On the other hand, the PI-uptake assay suggests melittin has a strong membranolytic effect causing ~100% permeability after only 15 min incubation (see Fig. 3), which is consistent with previous reports [43–45]. It is evident both peptides are active against *S. aureus* at similar concentrations but apparently with somewhat different modes of action. In the case of peptide-6 this may include self-aggregation/oligomerization as suggested by SPR assay and CD spectra (see above), which may play a role in membrane perturbation, as reported for membrane translocation peptides [46].

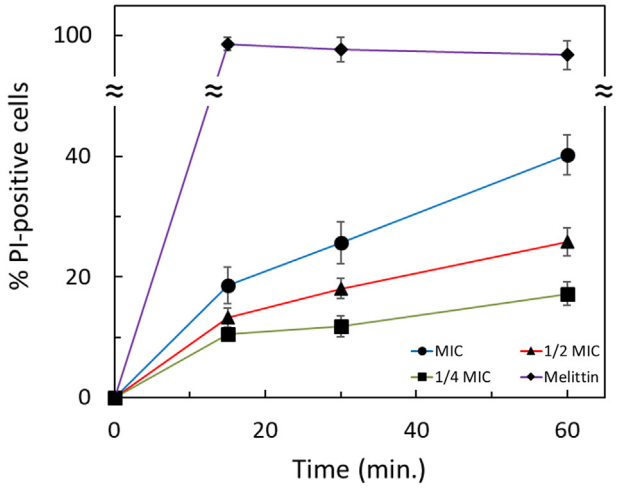

Fig. 3. Evaluation of the effect of peptide-6 on bacterial membrane integrity. Peptide was incubated with *S. aureus* ATCC 29213 (1×10^6 CFU/mL) for 60 min at concentrations equal to ¼ MIC, ½ MIC and MIC. Melittin was used as positive control at 5 μM. Data are expressed as the mean of % PI positive cells ± SEM of three independent experiments.

3.7. Cytotoxic effects

Both of the most active peptides (-1 and -6) were tested for their cytotoxic effects on different types of cells. Results on the MEC-1 cell line indicate that peptide-6 is somewhat more toxic than peptide-1. This is especially evident at > 50 μM concentration (see Fig. 5), although the IC_{50} value (44 μM) is significantly higher than the anti-*S. aureus*. Peptide-1 has a lower tendency to damage these cells with IC_{50} value at 105 μM (see Fig. 5). Both peptides were also examined for their mechanism of action to induce cell damage at concentrations ranging from 10 to 25 μM (MIC-MBC range). Dot-plots of the double stained cells show that MEC-1 cells are sensitive to apoptotic damage induced only by peptide-6, in a concentration-dependent manner (~40% and < 20% viable cells, respectively, at 10 and 25 μM, see Fig. S2, panels D–F). For comparison, peptide-1 (which showed a low but detectable cytotoxicity in the MTT assay), under the same conditions resulted in ~90% viable cells (see Fig. S2, panels B and C), comparable to untreated controls (see

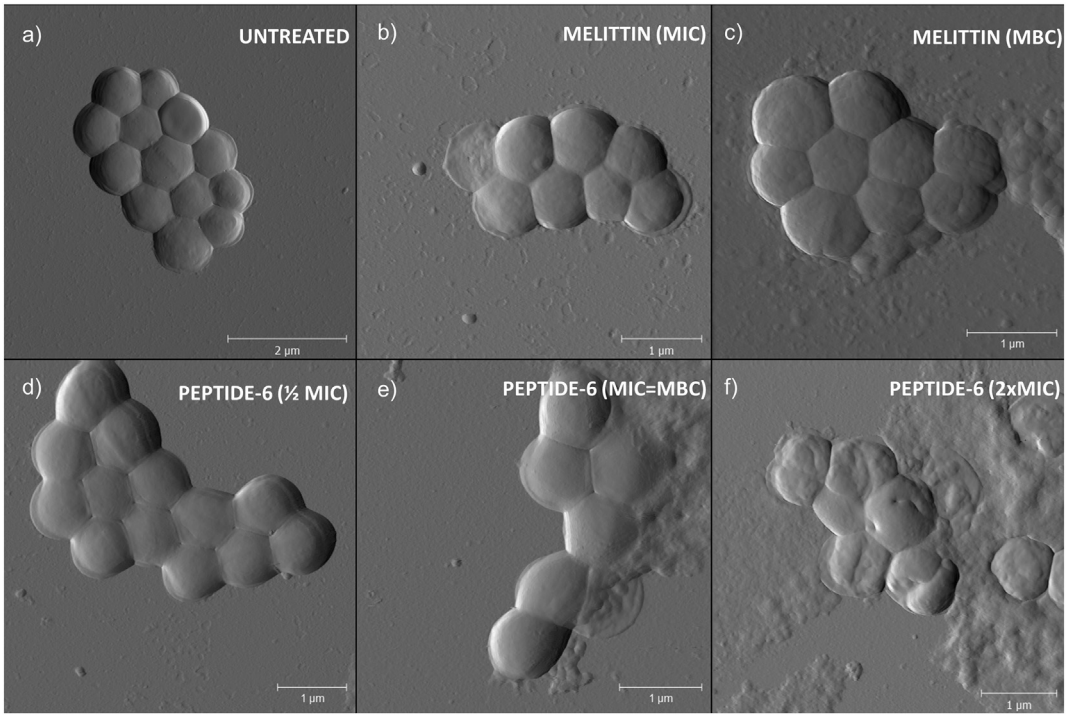

Fig. 4. AFM deflection (error) images of *S. aureus* ATCC 29213 cells. Bacteria were exposed to the peptide-6 at concentrations equivalent to the ½ MIC, MIC (MBC) and 2 × MIC.

Fig. S2, panel A, LR quadrant). Other peptides were not tested as they were found to be nontoxic in the MTT assay (see Fig. S3).

Although commonly used for studying cytotoxic effects [47], the MEC-1 cell line is in fact derived from tumor cells (B-chronic lymphocytic leukemia [30]). Neoplastic cells have features such as a more anionic membrane [48], that were previously correlated with enhanced peptides' tendency to interact with and disrupt cell membrane [49]. For this reason, the peptides' ability to induce cytotoxic effects was also tested on PBMC (resting circulating monocytes and lymphocytes derived from healthy donors) and for both peptide-1 and -6, the highest concentrations tested (100 μM) resulted in a cell viability > 80% (see Fig. 6). Based on these results, peptide-6 may be less toxic to normal than cancer-derived cells.

4. Conclusions

Six novel secretion peptides, derived from a targeted sequencing approach carried out simultaneously on five Ranidae species, were synthesized and one, identified from *R. arvalis*, showed significant activity against *S. aureus*, including a multi-drug resistant clinical isolate. This peptide appears to disrupt the bacterial membrane active concentrations and alters cell morphology at concentrations corresponding to 2 × MIC. Its activity is comparable to that of melittin, a potent membranolytic peptide derived from bee venom, but with somewhat different mechanism. Its mode-of-action may include self-aggregation/oligomerization at the membrane surface, which may be an initial step for membrane perturbation. The peptide toxicity against human circulating blood cells is negligible, with > 80% viable cells when tested at 100 μM concentration (considerably higher than to MIC value). It is somewhat more toxic against leukemia derived MEC-1 cells even

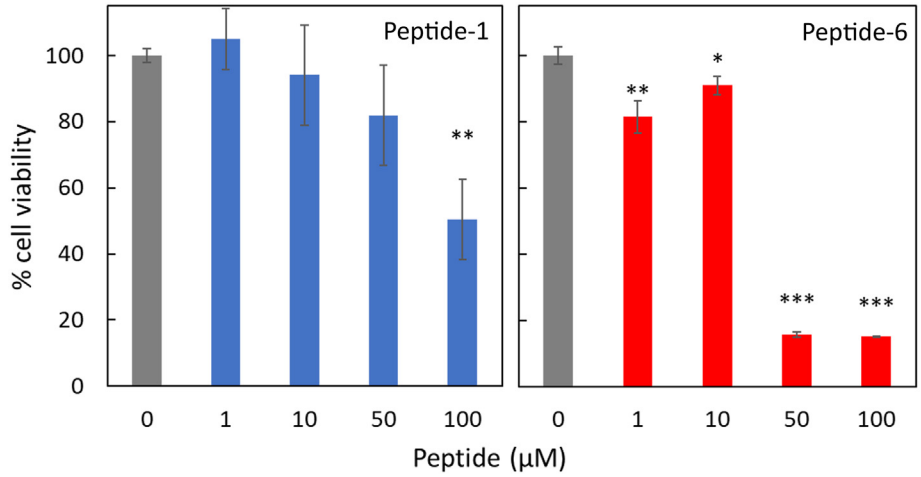

Fig. 5. Evaluation of cell viability in MEC-1 cells after exposure to peptide-1 and -6. Cells were exposed to 1, 10, 50, and 100 μM peptide for 24 h and evaluated by MTT assay. Viability is presented as % of the corresponding controls (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

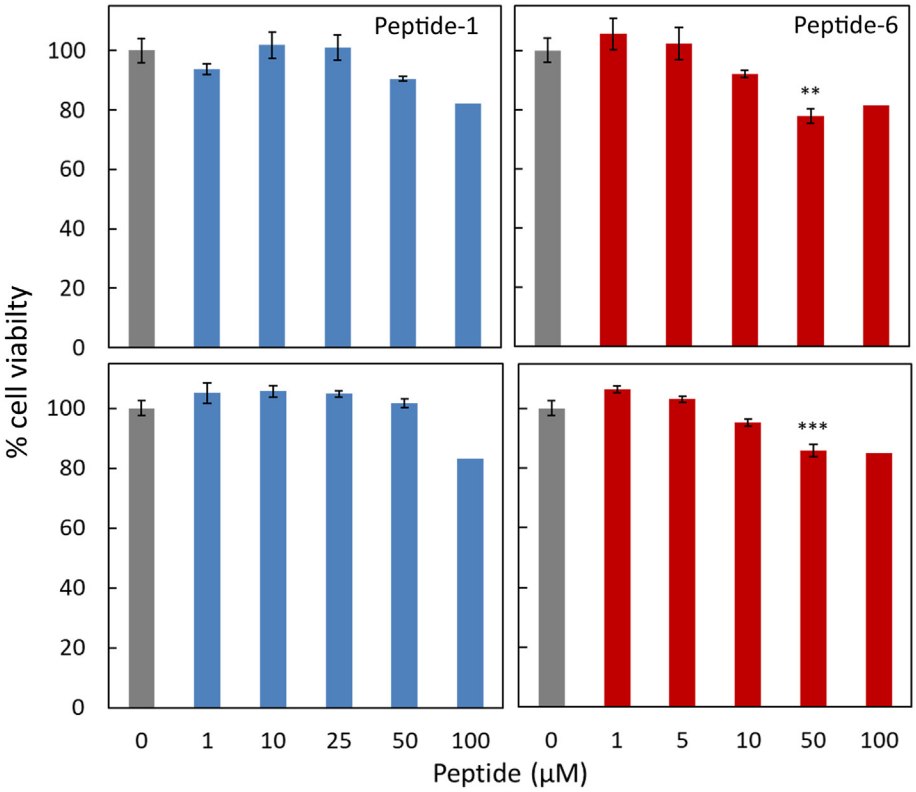

Fig. 6. Evaluation of cell viability in monocytes (lower panel) and lymphocytes (upper panel) after exposure to peptide-1 and -6. Cells were exposed to peptides up to 100 μM for 24 h and evaluated by MTT assay. Viability is presented as % of the corresponding controls (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

though the IC_{50} value remains several-fold higher compared to its MIC against *S. aureus*. In terms of possible biomedical application, the results are promising and suggest peptide-6 has a good selectivity for *S. aureus*, causing little damage to host cells at concentrations several-fold higher than the MIC values. All other peptides were only moderately or weakly active against bacteria, under the conditions used, in a salt/medium sensitive manner, and in general selective for Gram-positive strains.

It may thus be useful, in future analyses, to further study the effect of medium conditions on antimicrobial activity. Furthermore, as the activity did not correlate directly with membrane interactions (as assessed by CD), it may be useful to determine how membrane composition could affect activity. It may also be revealing to expand the tested bacteria, possibly including ones that tend to infect the host species, for possible narrow selectivity. It cannot however be excluded that the peptides have defense activities not involving direct bacterial inactivation.

Phylogenetic investigations revealed that peptide-6 does not pertain to any of the previously characterized anuran AMP families and is likely present as a single-copy gene in multiple species within the *Rana* genus. In the light of its appreciable antimicrobial activity, future investigations could be directed towards an orthologous AMP sequence identified in *R. temporaria*.

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. **Lucija Krce:** Methodology, Investigation, Formal analysis, Visualization. **Marco Gerdol:** Formal analysis, Investigation. **Sabrina Pacor:** Investigation. **Monica Benincasa:** Investigation. **Filomena Guida:** Investigation. **Ivica Aviani:** Methodology, Formal analysis. **Vedrana Čikeš-Čulić:** Methodology, Investigation. **Alberto Pallavicini:** Methodology, Formal analysis. **Ana Maravić:** Investigation. **Alessandro Tossi:** Methodology, Formal analysis, Writing - original draft, Writing - review & editing, Supervision.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbamem.2018.12.014.

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

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The third and final paper of the thesis depicts a different approach in obtaining peptides suitable for activity testing. The findings described in the first paper and other similar work have an evident value, however, they do not necessarily provide insight into the structural characteristics underlying the activity of the identified AMPs, and how to optimize this in the sequences of limited size. Such information would evidently be very useful, especially considering manufacturing costs involved in peptide synthesis, thus saving time and money in the searching for novel peptide antibiotics which could battle bacterial pathogens. For this reason, we also focused our efforts to linking peptides' biophysical properties with their biological potential. The laboratory where I carried out the largest part of my thesis work has developed methodologies for the design of AMPs with an optimized predicted balance between antimicrobial and cytotoxic effects, such as Mutator, a validated QSAR tool. In the past, Mutator was used by individually inputting peptide sequences, which resulted in some useful modifications of known peptides, but this was rather laborious and time consuming. We modified the algorithm by providing a filter that allowed screening of the entire Database of Anuran Defense Peptides to select a limited number of sequences with specified antimicrobial activities, and then used it to implement subtle changes (one or two amino acid substitutions) to obtain peptides with improved putative selectivity indices. In the end, we were successful in designing eight new peptides, out of which two were tested for antibacterial activity and host cell toxicity. Those peptides, named Dadapins, had rather high theoretical selectivity indices, which were confirmed experimentally. However, both peptides proved to have antibacterial activities that were sensitive to salt/media composition, thus underlining a pitfall in the described method, which relies on published but sometimes incomplete data, and which may however be overcome in the future. The problem does not relate so much to the algorithm in itself, but rather to the selection of peptides used as an input. Improved quality of the information present in the database, allowing for a more effective filtering process prior to inputting the sequences, could help prevent or at least help minimize it in the future.

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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

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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).

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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)	GLLRASSKWGRKYYVDLAGCAKA	+5	−1.42 (−0.04)	0.42	88.7
Dadapin-2	DM (V^8→K, A^{21}→L)	GLLRASSKWGRKYYVDLAGCLKA	+5	−0.95 (−0.03)	0.46	94.8
Dadapin-3	SM (V^8→K)	GLFGKSSKWGRKYYVDLAGCAKA	+5	−1.46 (0)	0.39	86.8
Dadapin-4	DHM (F^3→S, R^{11}→V)	GLSGKSSVWGVKYYVDLAGCAKA	+3	−0.86 (0.21)	0.18	89.9
Dadapin-5	DM (G^4→K, W^9→Q)	GLFKKSSVQGRKYYVDLAGCAKA	+5	−1.86 (−0.05)	0.32	94.6
Dadapin-6	DHM (A^7→K, R^{18}→L)	FLPKLFKKITKKNMAHIL	+6	0.31 (0.05)	0.38	94.9
Dadapin-7	DM (A^7→Q, R^{18}→L)	FLPKLFQKITKKNMAHIL	+5	0.53 (0.09)	0.37	94.9
Dadapin-8	DM (L^3→K, K^9→V)	AAKKGCWTVSIPPKPCF-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

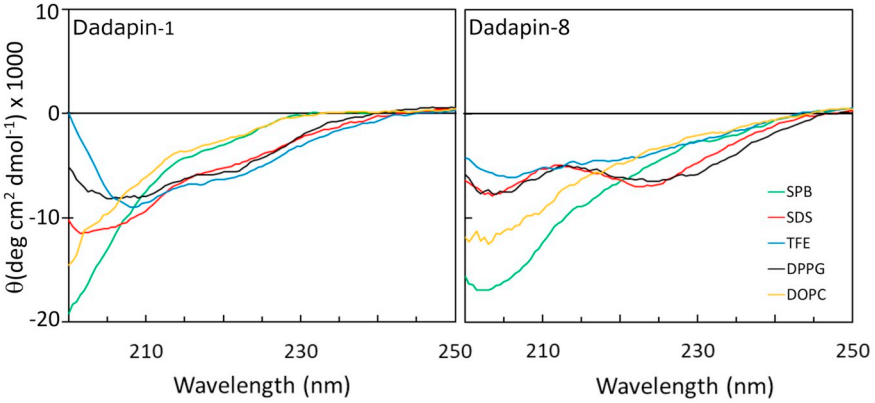

Fig. 1. CD spectra of Dadapin peptides under different conditions. Spectra are the accumulation of three scans carried out with 20 μM peptide in SPB, 10 mM SDS in SPB, 50% TFE, anionic LUVs in SPB (DPPG, 5 nM) and neutral LUVs in SPB (DOPC, 5 nM).

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

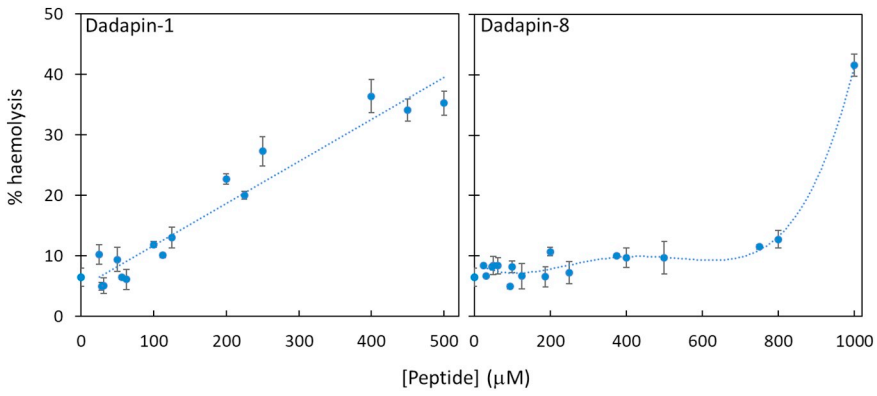

Fig. 2. Haemolytic activity of Dadapin peptides. Human erythrocytes were treated with peptides at increasing concentrations in PBS. Haemolysis was determined by measuring the absorbance of the supernatant at 450 nm after 1 h incubation of erythrocytes with each peptide at 37 °C and compared to the values 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.

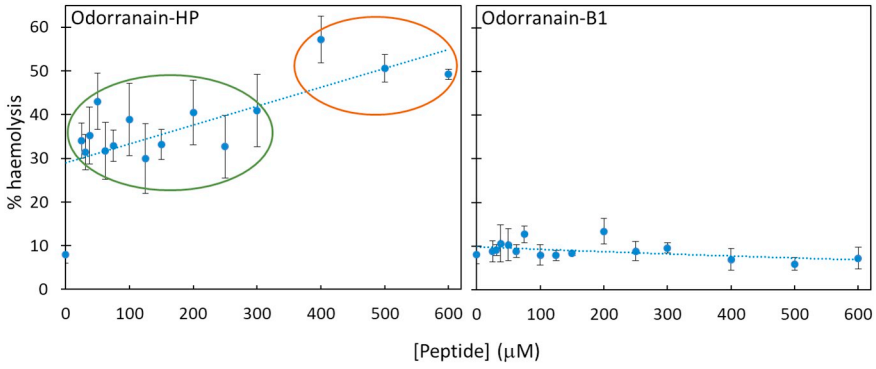

Fig. 3. Haemolytic activity of Odorranain peptides. Human erythrocytes were treated with peptides at increasing concentrations in PBS. Haemolysis was determined by measuring the absorbance of the supernatant at 450 nm after 1 h incubation of erythrocytes with each peptide at 37 °C and compared to the 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}.

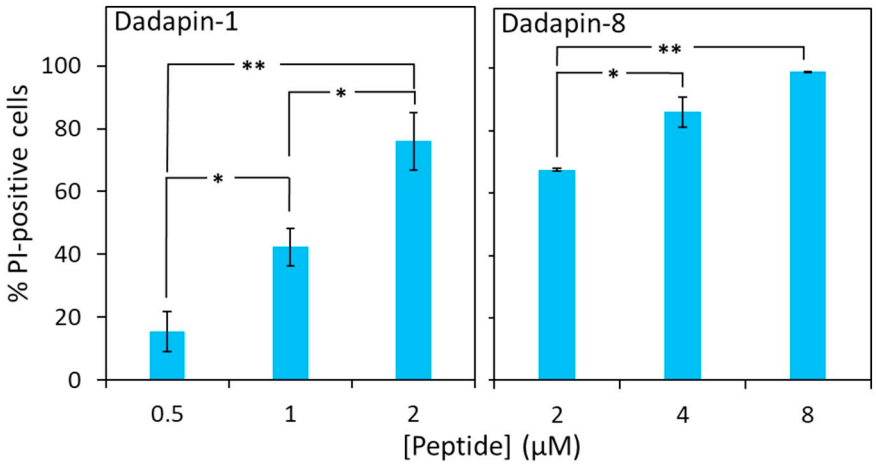

Fig. 4. Evaluation of the effect of Dadapin peptides on bacterial membrane integrity. Dadapin-1 was incubated with *S. aureus* (1×10^6 CFU/ml) and Dadapin-8 with *K. pneumoniae* (1×10^6 CFU/ml) for 15 min at concentrations equal to ¼ MIC, ½ MIC, MIC or 2 × MIC. Data represent % PI+ treated cells subtracted 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

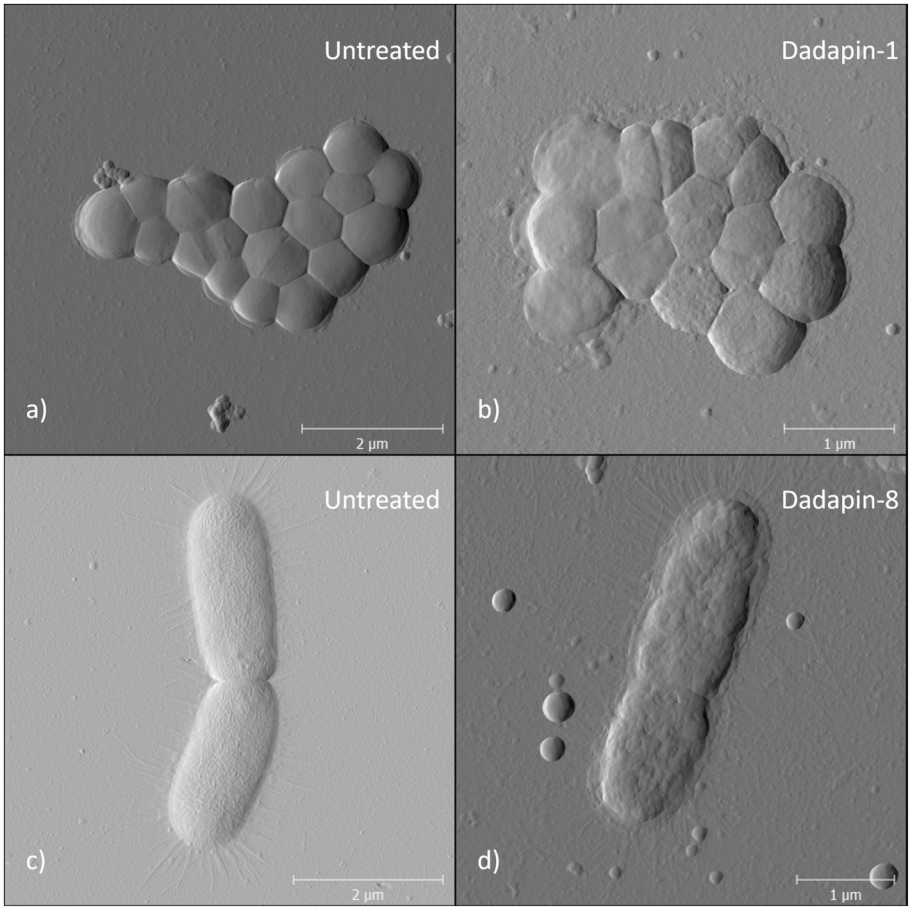

Fig. 5. AFM deflection images of untreated *S. aureus* [panel a)] and *K. pneumoniae* [panel c)] cells and in the presence of Dadapin-1 [panel b)] and Dadapin-8 [panel d)], respectively. Bacteria were exposed to the Dadapins at concentrations equivalent to 2 × MIC.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbamem.2019.01.017.

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IV OTHER WORK

During my period working on this thesis, I also collaborated in similar work on other peptides which included the development of the QSAR tool for the prediction of antibacterial activity for AMPs with a "Rana-box" domain [194]. This is a common cyclization motif at C-terminus of several peptides of anuran origin, which is assumed to have some relevance for the peptides' activity, possibly comparable to that of the preceding linear part of the sequence. This MIC prediction tool was based on the 'sideways asymmetry moment' (SAM), a more flexible profiling methodology compared to the commonly used hydrophobic moment. A two-descriptor model was developed by applying SAM to all amino acids in the segment before the Rana box (linear region) and to the cyclic region of Rana-box segment itself. The developed models are far from perfect as yet but can help distinguish peptides with poor and potent antibacterial activity. This was evaluated by synthesizing one peptide, named ranaboxin-1, which was predicted to have low MIC values and subsequently experimentally confirmed, thus providing an initial validation of the predication algorithm.

After that I worked on a family of peptides named kiadins. Initially, a tandem repeat of PGLa-H, one of the shortest α-helical peptides of anuran origin, was designed, which was followed with Val to Gly substitution resulting in kiadin-1 [81]. Both peptides proved to be quite potent, also against multi-drug resistant isolates of Gram-negative and Gram-positive species. They displayed a generally low toxicity for human circulating cells, and the selectivity for non-circulating cells was even more pronounced. Based on the results, it can be concluded that these peptides could serve as lead compounds to develop new and potent anti-infective agents.

Kiadin-2 to -6 were designed using a validated computational algorithm based on a training set of peptides active against *E. coli*. In some cases, this was followed by rational conformational alteration [195]. The sequences turned out remarkably similar to those of *di*PGLa-H and kiadin-1, so they were also included in the Kiadins family. In any case, the peptides had a varying content of Gly with different degree of conformational flexibility, which could in part be correlated with their varied activity spectrum and cytotoxicity. In general, kiadins with a higher proportion of Gly showed reduced antibacterial potential and caused less bacterial membrane alteration, as observed by flow cytometry and AFM. These observations correlated to the peptides' structural characteristics as observed by circular dichroism spectroscopy and predicted by molecular dynamics calculations. Furthermore, based on the

overall peptide sequences, together with the obtained results, we concluded that the amphipathic amino acid arrangement plays an equally important role in potency and selectivity of such peptides. Another interesting observation was that kiadins' potency did not necessarily correlate with permeabilizing capacity, but rather with the capacity to persist in the bacterial membrane. In conclusion, the information gathered on how structural modifications and amphipathic arrangement of residues affect peptide potency can be useful to improve design algorithms, thus contributing to the successful development of AMPs with potent antimicrobial activity.

I also collaborated in a quite different type of research, given the experience I gained in activity and toxicity assays, which concerned the biological activity of copper(II) and palladium(II) metal complexes. Such compounds are structurally far less complex compared to biomacromolecules such as AMPs, but their design in terms of antimicrobial or anticancer activity is equally demanding. In papers with Smrečki et al. [196–198] we have synthesized the aforementioned complexes with different ligands and tested them for their biological potential. My work with AMPs proved to be useful in this sense, allowing me to translate the acquired knowledge and to test their antimicrobial and antitumor potency. In most cases, the complexes proved to be inactive. However, the most recent paper describes the synthesis and evaluation of copper(II) complexes with N′-methylsarcosinamide [198], which proved to be selective *in vitro* for some cancer cell lines while completely inactive against microorganisms. At the same time, the complexes were nontoxic when tested against human lymphocytes.

V CONCLUSIONS AND FUTURE PERSPECITVES

The overall aim of this thesis was to develop new methodologies for identification and redesign of novel anuran AMPs. Both approaches (targeted DNA sequencing and peptide redesign with Mutator) proved to be successful with the former providing means for high-throughput simultaneous identification of novel anuran AMPs from different frog species belonging to different families. In recent years, related approaches using available transcriptomic data have been proposed for high-throughput peptide identification but did not include the use of forward degenerate primers designed based on highly conserved regions of AMP precursors (e.g. signal peptide and anionic propeptide regions). More than 100 potential AMPs have been identified in this way out of which 29 were novel sequences validating this approach and confirming its effectiveness. As discussed in paper I, all peptides have been identified in species pertaining to Ranidae family, whereas no AMPs were found in Hylidae and Bombinatoridae. There could be several reasons for this, most notably unfavorable phylogenetic relationships between assembled transcripts and the species used for total RNA extraction and the excessive transcript size with respect to the sequencing platform that was used. In the future, these findings (together with other observations depicted in paper I) should be taken into consideration when conducting similar experiments to increase the peptide identification yield. The approach presented in this paper can be applied to any species with conserved signal peptide and/or propeptide regions associated to AMPs. It would also be interesting to conduct such investigations in species where no AMPs have been identified so far, for example prokaryotes that have been the source of peptide antibiotics in the past. Ribosomally or non-ribosomally synthesized AMPs are often modified after translation (anuran AMPs are often amidated at the C-terminus), thus improving stability and activity. With this method, no such information can be obtained, and the experiment could be designed to couple the described approach with mass spectrometry techniques to gain insights about such modifications, potentially indicating more potent AMPs.

Six peptides, out of 29 identified in this way, have been extensively characterized (as shown in paper II) for antimicrobial activity, toxicity towards host cells and mode of action. In general, peptides proved to be non-toxic with moderate to limited antibacterial activity for Peptides-1 to -5. Peptide-6 showed good activity against *S. aureus* with limited or no toxicity on MEC-1 cells, monocytes and lymphocytes and was further tested for its particular mode of action. Based on flow cytometry data and AFM imaging the peptide proved to be membrane active and pore forming, whereas CD data and surface plasmon resonance indicated that the peptide could

oligomerize/self-aggregate in the interaction with the artificial membrane. Potentially, the peptide/s can also be tested for their ability to interfere with some intracellular processes such as the complex machinery involved in protein synthesis (transcription/translation). The activity of helical peptides is in most cases linked exclusively to membrane perturbation, although certain peptide classes are known to utilize both membrane permeabilizing and non-lytic mode of action. In any case, these experiments are an excellent first step to test the *in vitro* potency of selected peptides. However, the next step, for these or some of the more potent peptides, should be *in vivo* experiments on lab animals, preferably mice. Such pre-clinical trials and pharmacokinetic studies can determine if the peptide is suitable for systemic use and possible human application which is, very often, the final goal of different AMPs studies. In fact, similar research is currently in the progress since recently we have identified novel AMPs from parasitic eukaryotic species. These are quite potent against Gram-negative bacteria, including multi-drug resistant isolates, with limited toxicity to human lymphocytes. The structure and mode of action has been evaluated by CD, flow cytometry and AFM imagining, and *in vivo* experiments are soon to be carried out on bacteria infected mice. Data obtained from these experiments should provide adequate information regarding peptide stability and potency *in vivo*, making the grounds (or not) for future clinical testing.

Concerning peptide redesign with the Mutator tool, applying a filtering process on the DADP anuran bioactive peptides database and subtle modifications to a generated sub-set of previously identified sequences resulted in eight new peptides with theoretically high selectivity index, as shown in paper III. Out of these, two have been selected for synthesis and comparison with the parent peptide sequences, showing reduced toxicity towards human red blood cells and increased antibacterial potency. Both peptides proved to be membrane active, as confirmed by flow cytometry and AFM imaging. However, both peptides also were shown to be quite salt/medium sensitive, with a significant decrease in activity in full growth medium, underlining *i*) the complexity of peptide design for potential biomedical applications and *ii*) some pitfalls of QSAR approach. The descriptor used for the prediction of AMP activity is only as good as the peptide set used to build it. In other words, the tool could potentially be improved by incorporating changes into the original Mutator algorithm by assembling an AMP dataset containing only peptides with robust activity. Some regularities/patterns from this set could then be incorporated into the original algorithm to build additional restrictions, and hopefully, generate a set of peptides with high selectivity index and lower sensitivity to salt/medium/serum components.

VI APPENDICES

This chapter depicts supplementary material to the papers that constitute the main part of the thesis. Given the fact that paper I has been published in BMC Genomics under open access policy, its contents, including supplementary material, are freely available online at https://bmcgenomics.biomedcentral.com/articles/10.1186/s12864-018-5225-5. Supplementary materials to papers II and III, which are currently not available online free of charge, can be found below.

1 Supplementary material to paper II

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T. Rončević, L. Krce, M. Gerdol, S. Pacor, M. Benincasa, F. Guida, I. Aviani, V. Čikeš-Čulić, A. Pallavicini, A. Maravić, A. Tossi, Membrane-active antimicrobial peptide identified in Rana arvalis by targeted DNA sequencing, Biochim. Biophys. Acta BBA - Biomembr. 1861 (2019) 651–659. doi:10.1016/j.bbamem.2018.12.014.

Membrane-active antimicrobial peptide identified in *Rana arvalis* by targeted DNA sequencing.

Tomislav Rončević[a,*], Lucija Krce[a], Marco Gerdol[b], Sabrina Pacor[b], Monica Benincasa[b], Filomena Guida[b], Ivica Aviani[a], Vedrana Čikeš-Čulić[c], Alberto Pallavicini[b], Ana Maravić[d], Alessandro Tossi[b]

[a]Department of Physics, Faculty of Science, University of Split

[b]Department of Life Sciences, University of Trieste

[c]Department of Medical Chemistry and Biochemistry, School of Medicine, University of Split

[d]Department of Biology, Faculty of Science, University of Split

*Corresponding author (E-mail: troncevic@pmfst.hr; Tel:+385-21-619-230)

Supporting information

Figure S1. Analytical RP-HPLC of Ranidae peptides.

Table S1. Sequence identity among the propeptide and 3'UTRs regions of the mRNAs encoding *Rana temporaria* temporins.

Table S2. α-helix percentage of Ranidae peptides in different environments.

Table S3. Antibacterial activity (μM) of Ranidae peptides in 20% MH medium.

Figure S2. Dot-plot of the double stained MEC-1 cells after treatment with peptide-1 and -6.

Figure S3. Toxic effect of Ranidae peptides (2-5) on MEC-1 cell line evaluated by MTT assay.

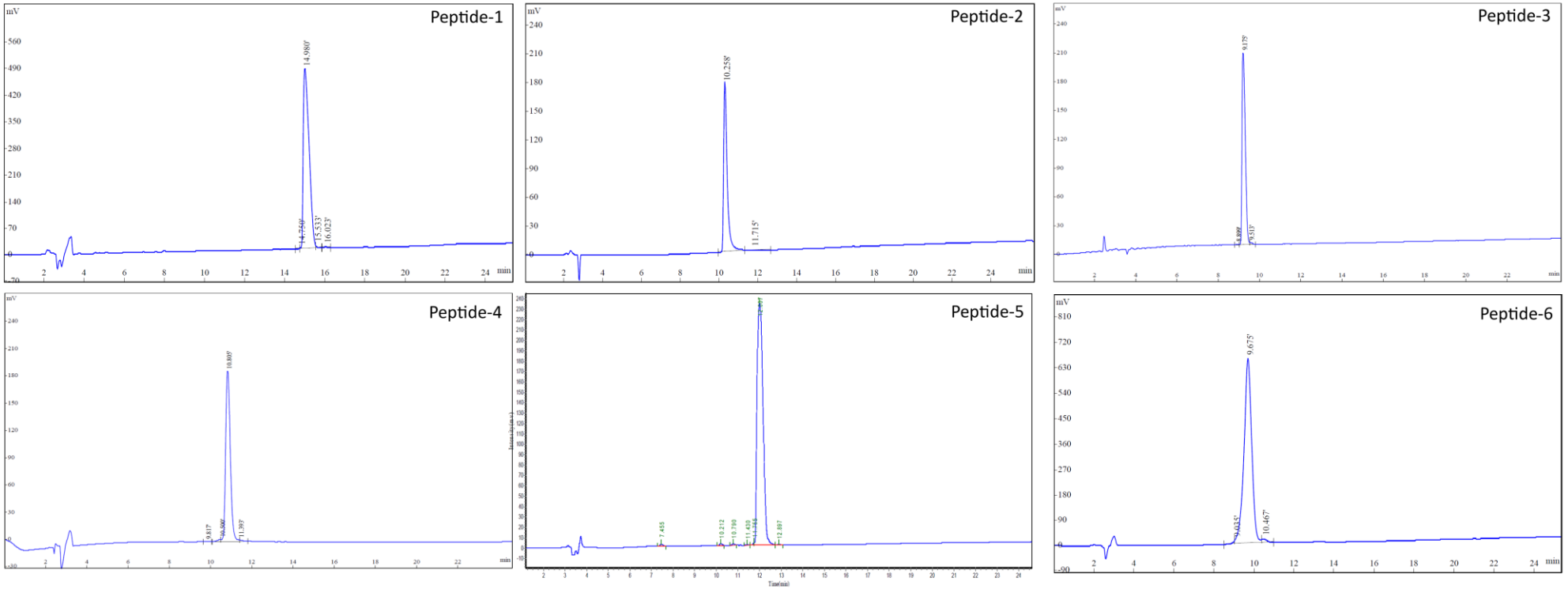

Figure S1. Analytical column (C18, 5 µm, 110 Å, 4.6 x 250 mm) was used with 25–85% acetonitrile/0.1% TFA gradient in 25 min with flow of 1.0 mL/min.

Table S1. Sequence identity among the a) propeptide and b) 3'UTRs regions of the mRNAs encoding *Rana temporaria* temporins, including the two newly identified and *de novo* synthesized peptides rtemp_4.3_210 and rtemp_4.6_20.

a)

		1	2	3	4	5	6	7	8
rtemp_4.3_210 (peptide1)	1		92,78	100,00	90,62	89,58	76,92	94,79	90,00
rtemp_4.6_20 (peptide2)	2	92,78		92,78	87,63	86,60	76,07	91,75	94,00
rtemp_1.10_80 (temporin-A)	3	100,00	92,78		90,62	89,58	76,92	94,79	90,00
rtemp_1_852 (temporin-B)	4	90,62	87,63	90,62		95,83	70,94	89,58	85,00
rtemp_2_141 (temporin-C)	5	89,58	86,60	89,58	95,83		70,09	90,62	84,00
rtemp_107_11_(temporin-F)	6	76,92	76,07	76,92	70,94	70,09		76,07	77,97
rtemp_4.21_24 (temporin-L)	7	94,79	91,75	94,79	89,58	90,62	76,07		89,00
rtemp_4.7_33 (novel)	8	90,00	94,00	90,00	85,00	84,00	77,97	89,00	

b)

		1	2	3	4	5	6	7	8
rtemp_4.3_210 (peptide1)	1		87,37	87,23	75,53	74,47	96,70	96,70	95,60
rtemp_4.6_20 (peptide2)	2	87,37		81,25	70,83	69,79	86,17	86,32	91,58
rtemp_1.10_80 (temporin-A)	3	87,23	81,25		75,27	74,19	84,04	87,23	85,11
rtemp_1_852 (temporin-B)	4	75,53	70,83	75,27		96,34	72,34	75,53	75,53
rtemp_2_141 (temporin-C)	5	74,47	69,79	74,19	96,34		71,28	74,47	74,47
rtemp_107_11 (temporin-F)	6	96,70	86,17	84,04	72,34	71,28		93,41	92,31
rtemp_4.21_24 (temporin-L)	7	96,70	86,32	87,23	75,53	74,47	93,41		94,51
rtemp_4.7_33 (novel)	8	95,60	91,58	85,11	75,53	74,47	92,31	94,51	

Table S2. α-helix percentage of Ranidae peptides in different environments. Measurements were 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).

	% helix				
Peptide	**SPB**	**SDS**	**TFE**	**DPPG**	**DOPC**
Peptide-1	<5	30	55	60	20
Peptide-2	<5	50	35	75	45
Peptide-3	10	10	20	10	10
Peptide-4	5	15	15	5	<5
Peptide-5	<5	<5	<5	10	<5
Peptide-6	<5	25	35	40	40

Table S3. Antibacterial activity (μM) of Ranidae peptides against Gram-negative and Gram-positive bacterial strains in 20% MH medium.

	Peptide-1		**Peptide-2**		**Peptide-5**		**Peptide-6**	
Bacterial strains	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
S. aureus ATCC 29213	4	4	8	16	16	16	1	1
S. aureus c.i.	32	32	64	64	64	64	4	4
E. coli ATCC 25922	16	32	64	64	32	32	8	8
A. baumannii ATCC 19606	16	16	64	64	>64	/	16	16

Peptide-3 and -4 showed no activity even when tested in 20% MH medium.
E. faecalis ATCC 29212 did not grow in medium with reduced concentration of nutrients (20% MH).

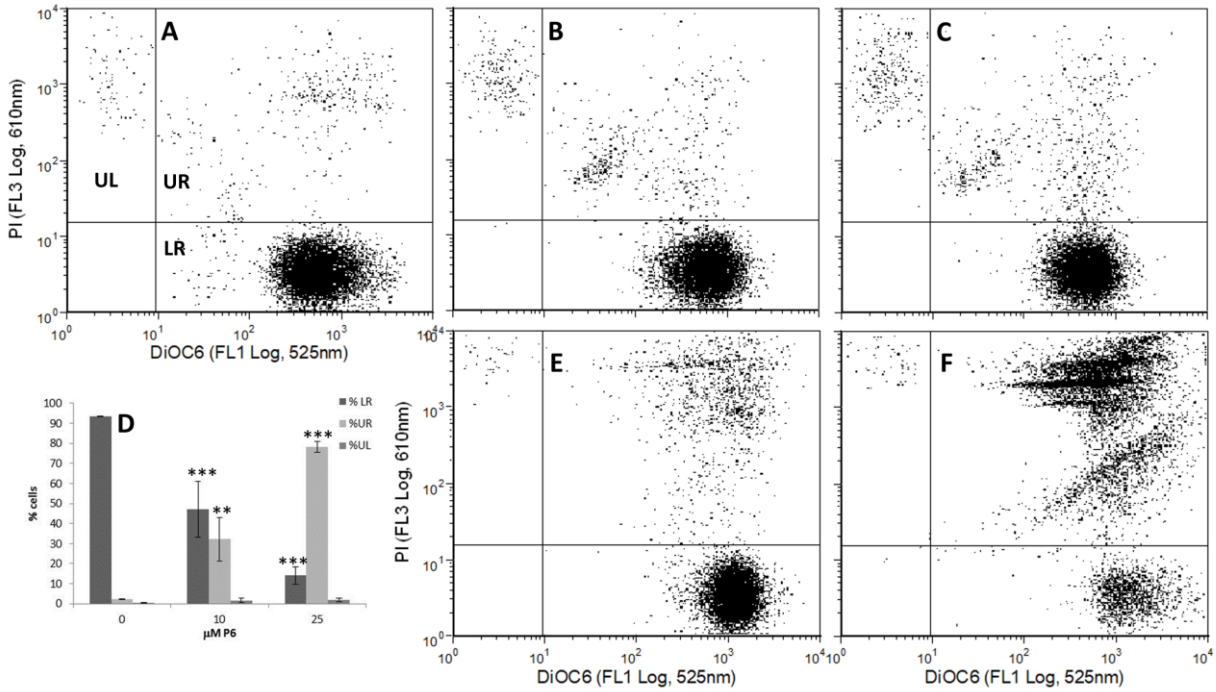

Figure S2. Apoptotic-necrotic assay of MEC-1 treated cells. Dot-plot of double stained untreated control with viable cells in LR quadrant (DiOC6+/PI-), late apoptotic (UR quadrant, DiOC6+/PI+) and necrotic (UL quadrant, DiOC6-/PI+) (panel A). Representative dot-plots of cells treated for 30 min with peptide-1 at 10μm (B) or 25μM (C), and peptide-6 at 10μm (E) or 25μM (F). Histogram of panel D represents mean values ± SEM of % of P6 treated cells in each quadrant. ***p<0,001, **p<0,01 values statistically significant from untreated controls, ANOVA Student-Newman-Keuls test.

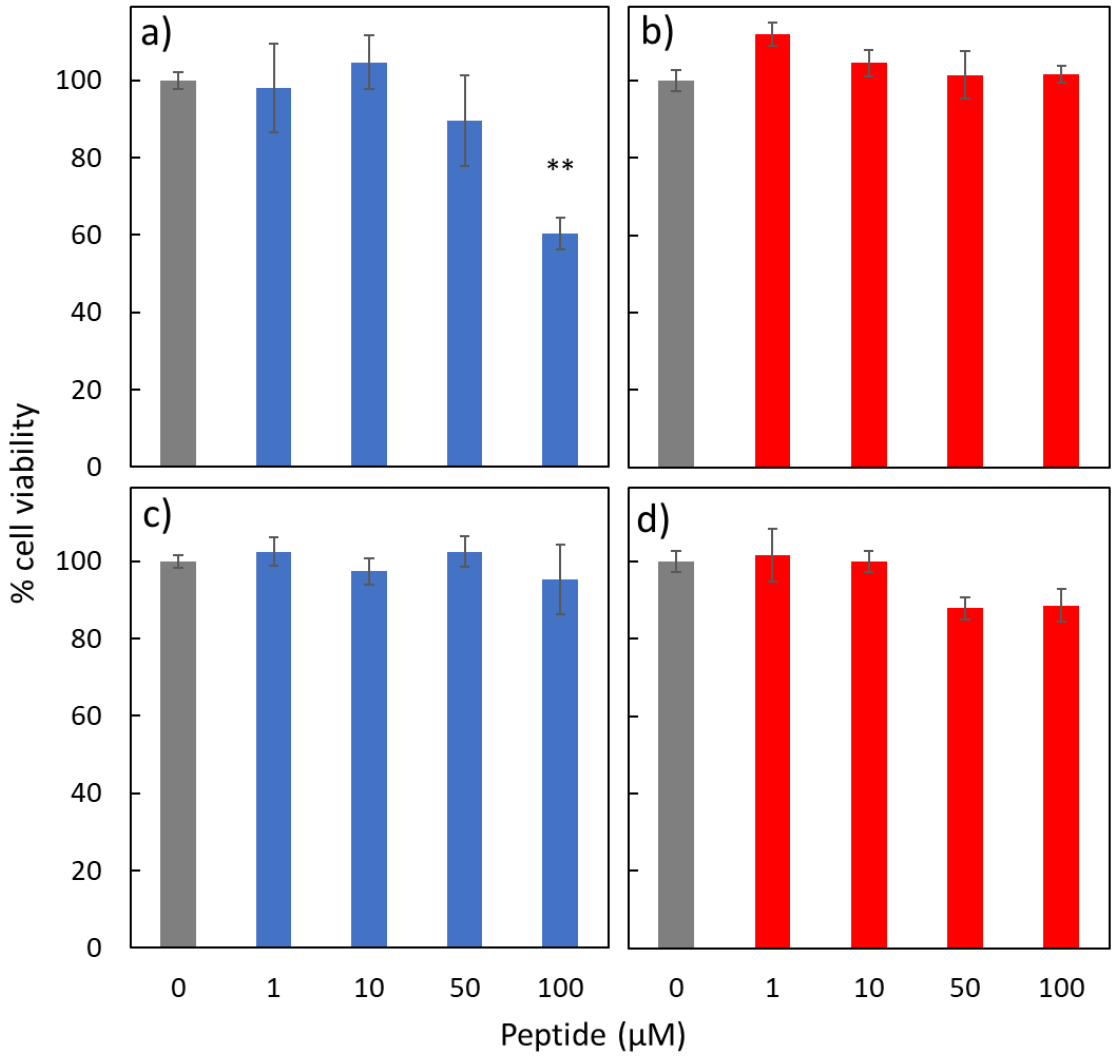

Figure S3. Evaluation of cell viability in MEC-1 cells after exposure to a) peptide-2, b) peptide-3, c) peptide-4 and d) peptide-5. Cells were exposed to 0, 1, 10, 50, and 100 µM for 24 h and evaluated by MTT assay. Viability is presented as % of the corresponding controls (*p<0.05, **p<0.01, ***p<0.001).

2 Supplementary material to paper III

Reproduced from:

T. Rončević, D. Vukičević, L. Krce, M. Benincasa, I. Aviani, A. Maravić, A. Tossi, Selection and redesign for high selectivity of membrane-active antimicrobial peptides from a dedicated sequence/function database, Biochim. Biophys. Acta BBA - Biomembr. 1861 (2019) 827–834. doi:10.1016/j.bbamem.2019.01.017.

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] and 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

*Corresponding author (E-mail: troncevic@pmfst.hr; Tel:+385-21-619-230)

Supporting information

Table S1. Data set of 62 peptides of anuran origin analyzed using Mutator.

Figure S1. Analytical RP-HPLC of Dadapin peptides.

Figure S2. Analytical RP-HPLC of Odorranain peptides.

Table S1. Data set of 62 peptides of anuran origin analyzed using Mutator.

#	Peptide	DADP ID	Sequence
1	Dermaseptin-H4	SP_Q1EJP5	GLWSTIKNVGKEAAIAAGKAALGAL
2	Dermaseptin-H5	SP_2676	GLWSTIKNVGKEAAIAAGKAVLGSL
3	Amolopin-2f	SP_C5H0D7	FFPIVGKLLFGLSGLL
4	Andersonin-Y1	SP_2843	FLPKLFAKITKKNMAHIR
5	Amolopin-2e	SP_C5H0D6	FLPIAGKLLSGLSGLL
6	Gaegurin-6-RN	SP_C0ILG2	FIGPVLKIAAGILPTAICKIFKKC
7	Brevinin-1Bb	SP_P82834	FLPAIAGMAAKFLPKIFCAISKKC
8	Maximin H2	SP_B5L106	ILGPVLSMVGSALGGLIKKI
9	Amolopin-2a	SP_A0SN45	FLPIVGKLLSGLSGLL
10	Grahamin-2	SP_P85070	GLLSGILGAGKHIVCGLSGLC
11	Grahamin-1	SP_P0C2A4	GLLSGILGAGKNIVCGLSGLC
12	Amolopin-2h	SP_C5H0D9	FFPIVGKLLFGLFGLL
13	Gaegurin-6-RN	SP_C0ILJ3	FLGPIIKIATGILPTAICKFLKKC
14	Brevinin-1VLc	SP_2591	FLPVIASVAAKVLPKVFCFITKKC
15	Brevinin-1BLa	SP_2603	FLPAIVGAAAKFLPKIFCAISKKC
16	Brevinin-1BLc	SP_2604	FLPIIAGIAAKFLPKIFCTISKKC
17	Amolopin-2g	SP_C5H0D8	FFPIVGKLLSGLSGLL
18	Esculentin-1-OA6	SP_2920	GLFSKFAGKGIKNFLNKGVKHIGKE
19	Temporin-ALh	SP_2678	FLPIVGKLLSGLSGLS
20	Histone 2A	SP_2965	TRSSRAGLQFPVGRVHRLLRK
21	Bombinin-like peptide 3	SP_P29004	GIGAAILSAGKSALKGLAKGLAEHF
22	Nigrocin-1-OW4	SP_2942	GILSGVLGMGKKIVCGLRGLC
23	Ranalexin	SP_P39084	FLGGLIKIVPAMICAVTKKC
24	Phylloseptin-2	SP_P84567	FLSLIPHAINAVSTLVHHF
25	Phylloseptin-2	SP_P84930	FLSLIPHIATGIAALAKHL
26	Dybowskin-4	SP_2687	VWPLGLVICKALKIC
27	Brevinin-1-OR9	SP_2897	ILPFVAGVAAMEMEHVYCAASKKC
28	Temporin-RN	SP_C0ILK2	FFPLLFGALSSHLPKLF
29	Odorranain-HP	SP_A7YL71	GLLRASSVWGRKYYVDLAGCAKA
30	Preprotemporin-1Oa1	SP_A3KD26	FLPLLASLFSRLL
31	Brevinin-1CSa	SP_2658	FLPILAGLAAKIVPKLFCLATKKC

32	Amolopin-1b	SP_A0SN42	FLPLAVSLAANFLPKLFCKITKKC
33	Caerin 1.6	SP_2996	GLFSVLGAVAKHVLPHVVPVIAEKL
34	Caerin-1.6	SP_P62547	GLFSVLGAVAKHVLPHVVPVIAEK
35	Brevinin-1E	SP_P32412	FLPLLAGLAANFLPKIFCKITRKC
36	Maximin H1	SP_P83080	ILGPVISTIGGVLGGLLKNL
37	Brevinin-1E	SP_P32412	FLPLLAGLAANFLPKIFCKITRKC
38	Brevinin-1BYa	SP_P84111	FLPILASLAAKFGPKLFCLVTKKC
39	Brevinin-1CHa	SP_2651	FLPIIAGVAAKVLPKLFCAITKKC
40	Brevinin-1CHb	SP_2652	FLPVIAGLAAKVLPKLFCAITKKC
41	Nigrocin-1-OW5	SP_2943	GILGNIVGMGKQVVCGLSGLC
42	Odorranain-W1	SP_A6MBS8	GLFGKSSVWGRKYYVDLAGCAKA
43	Nigrocin-1-OA3	SP_2935	GIFLKVLGVGKKVLCGVSGLC
44	Brevinin-1-RAA10	SP_D2K8J2	FLPAVIRVAANVLPTAFCAISKKC
45	Brevinin-1-OR10	SP_2898	FLPAVLLVATHVLPTVFCAITRKC
46	Odorranain B1	SP_A6MBD6	AALKGCWTKSIPPKPCFGKR
47	Odorranain-H2	SP_A6MBN4	GIFGKILGVGKKVLCGLSGVC
48	Lividin-7a	SP_C3RSZ8	GILSGILGVGKKLVCGLSGLC
49	Nigrocin-1-OA2	SP_2934	GIFGKILGVGKKTLCELSGMC
50	Ascaphin-8	SP_P0CJ32	GFKDLLKGAAKALVKTVLF
51	Brevinin-1Bd	SP_P82836	FLPAIAGVAAKFLPKIFCAISKKC
52	Brevinin-1Be	SP_P82837	FLPAIVGAAAKFLPKIFCVISKKC
53	Brevinin-1HSa	SP_P0C8S7	FLPAVLRVAAKIVPTVFCAISKKC
54	Brevinin-1PTa	SP_P0C8T1	FMGGLIKAATKIVPAAYCAITKKC
55	Brevinin-1VLa	SP_2590	FLGAIAGVAAKFLPKVFCFITKKC
56	Brevinin-1Ya	SP_2605	FLPVIAGVAANFLPKLFCAISKKC
57	Brevinin-1Yb	SP_2606	FLPIIAGAAAKVVQKIFCAISKKC
58	Brevinin-1AUa	SP_2622	FLPILAGLAAKLVPKVFCSITKKC
59	Brevinin-1AUb	SP_2623	FLPILAGLAANILPKVFCSITKKC
60	Temporin-1Sa	SP_2689	FLSGIVGMLGKLF
61	Brevinin-1SPa	SP_2692	FFPIIAGMAAKLIPSLFCKITKKC
62	Brevinin-1SPd	SP_2694	FFPIIAGMAAKVICAITKKC

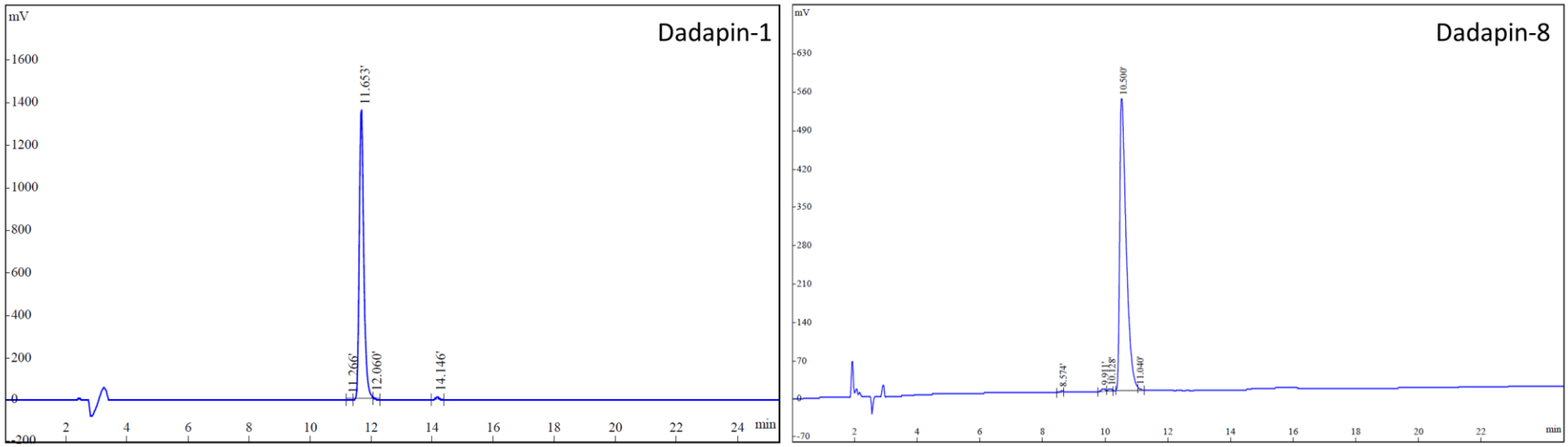

Figure S1. Analytical column (C18, 5 µm, 110 Å, 4.6 x 250 mm) was used with 20-50 % acetonitrile/0.1% TFA gradient in 25 min with flow of 1.0 mL/min.

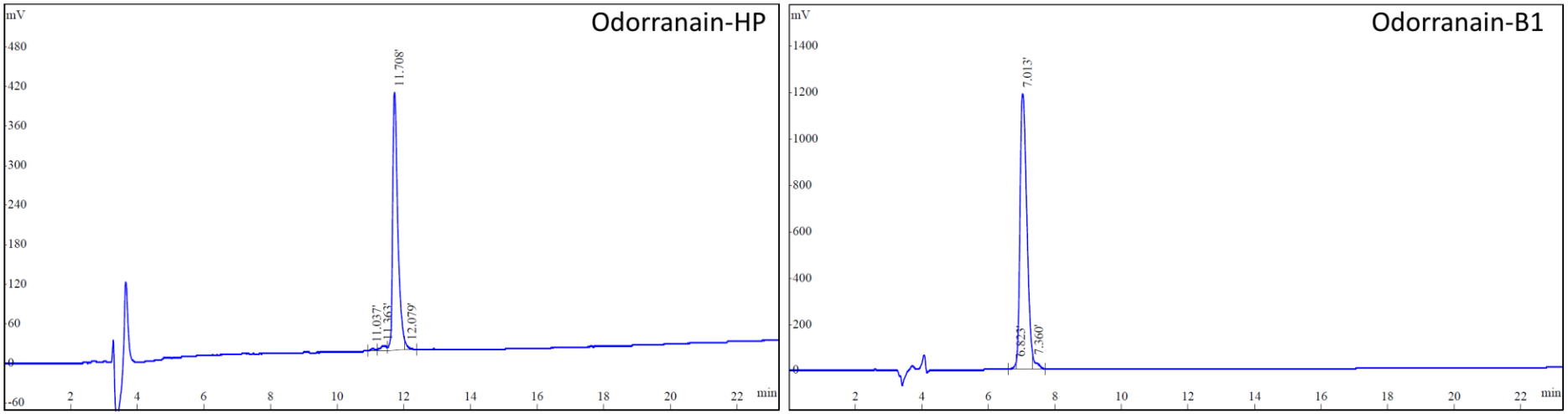

Figure S2. Analytical column (C18, 5 µm, 110 Å, 4.6 x 250 mm) was used with 10-70 % acetonitrile/0.1% TFA gradient in 25 min with flow of 1.0 mL/min.

CURRICULUM VITAE

TOMISLAV RONČEVIĆ

Date of birth: 17th October 1985

Place of birth: Zadar, Croatia

Nationality: Croat

EDUCATION

- Ph.D. student at the Doctoral school of Biophysics (2014 – present)
  Faculty of Science, University of Split, Split, Croatia
- M.Sc. in Biology and Chemistry (2004 - 2014)
  Faculty of Science, University of Zagreb, Zagreb, Croatia

EMPLOYMENT

- Doctoral fellow (February 2015 - present)
  Faculty of Science, University of Split, Split, Croatia

  Work on the Croatian Science Foundation project IP-8481 "Biophysical Design of Antimicrobial peptides and Innovative Molecular Descriptors" (PI: Prof. Damir Vukičević, Ph.D.; formerly Prof. Davor Juretić, Ph.D.)

AWARDS AND GRANTS

- Student Quorum of the University of Split project: "Antimicrobial peptides – new ways of dealing with multi-resistant clinical pathogens and cancer cells" (2017)
- Erasmus + grant (2017)
- ICGEB travel and local hospitality grant (2016)
- Faculty of Science (Split) award for the best scientific paper in the previous year (2016)

LIST OF PUBLICATIONS

[1] T. Rončević, D. Vukičević, L. Krce, M. Benincasa, I. Aviani, A. Maravić, A. Tossi, Selection and redesign for high selectivity of membrane-active antimicrobial peptides from a dedicated sequence/function database, Biochim. Biophys. Acta BBA - Biomembr. 1861 (2019) 827–834. doi:10.1016/j.bbamem.2019.01.017.

[2] N. Smrečki, T. Rončević, O. Jović, B.-M. Kukovec, A. Maravić, G. Gajski, V. Čikeš-Čulić, Copper(II) complexes with N′-methylsarcosinamide selective for human bladder cancer cells, Inorganica Chim. Acta. 488 (2019) 312–320. doi:10.1016/j.ica.2019.01.013.

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