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Insights into the Antimicrobial Activities of Unusual Antimicrobial Peptide Families from Amphibian Skin

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Abstract

Frog's skin secretions are known to present peculiar characteristics involving an arsenal of bioactive molecules. These organisms, in response to stress, injury or predator attack, release a viscous toxic secretion through granular glands containing biogenic amines, alkaloids, steroids, proteins and also peptides. Among such compounds, the antimicrobial peptides (AMPs) are responsible to play an important role in amphibian first-line defense against pathogenic microorganisms such as Gram-negative and positive bacteria, fungi and virus. In amphibians, AMPs have been isolated from different species and functionally studied, presenting not only antimicrobial but also antitumor, antifungal, anti-protozoa and spermicidal activities. However, a large number of AMPs have also shown cytotoxic activities against mammalian cells. In order to develop novel anti-infective drugs with low side effects, recent research has also been done to describe novel frog AMPs with different structural patterns. In this context, this review will focus on the antimicrobial activities of nine recently discovered amphibian AMPs including phylloseptins, nigrocins, japonicins, palustrins, parkerins, jingdongins, medusins, limnonectins and hylaranins. The biochemical properties will be discussed, as well as their possible applications in human health as new alternatives to conventional medicines.

Keywords: Amphibian defense; Antimicrobial peptides; Biotechnological compounds; Human health

Introduction

In recent years, the increase in bacterial resistance has become a major concern for human health. It poses a major challenge in the discovery and improvement of effective drugs, and there has been great interest in novel bioactive molecules such as the antimicrobial peptides (AMPs) [1,2]. AMPs are small, linear or cyclic cationic molecules, composed of few amino acid residues, usually 8 to 50 residues, which are known to present amphipathic structures composed of hydrophobic and positively charged regions. AMPs have been found in several species of vertebrates and invertebrates as well as in plants [3,4], being divided into major groups based on their length, linearity, charge, structural conformation (a-helix, b-sheets and coil) and their predisposition (unusual or not) for particular repetition of some amino acid residues [5]. In addition, AMPs present a broad spectrum of activities against bacteria, fungi and viruses, making them an unconventional alternative for production of effective drugs against microorganisms [2].

Amphibians were among the first organisms to form a connecting link between water and land and, for this reason; they developed different strategies to survive in a variety of conditions. Their skin has basic functions such as water regulation and respiration. The mucus gland secretion helps to maintain a moist skin surface and prevents mechanical damage to skin [1]. Moreover, the moist amphibian's skin is also known to be an ideal habitat for microbial growth [6]. These factors contributed to the evolution process of an innate immune defense system based on secretions containing a range of myotropic peptides, mast cell degranulating peptides, neuroendocrine peptides and a diverse array of AMPs, which are synthetized by granular glands [3,7]. These chemical compounds provide protection against microorganisms and predators [8]. The aforementioned secretions can be divided into different categories such as biogenic amines, steroids, alkaloids and proteinaceous compounds, which include the defensive peptides [7]. Several families of antimicrobial peptides have been isolated from amphibians and some of them such as magainins, temporins, bombinins and brevinins have been widely studied, been also subject of recent review articles. In this context, this review focuses on the antimicrobial properties of nine unusual AMPs discovered in the last 15 years, including phylloseptins, nigrocins, japonicins, palustrins, parkerins, jingdongins, medusins, limnonectins and hylaranins, and also discusses the possible applications of these AMPs in human health.

Amps Synthesis in the Granular Glands of Frogs

Cutaneous glands are responsible for 90% of the gas exchange in amphibians. They can be divided into two types: 1) mucous glands, which are evenly distributed over the body and secrete sticky substances that prevent desiccation and facilitate cutaneous respiration; 2) granular (venomous) glands, which are present in the dermis and may be distributed in compact groups, characterized by lumps on the skin at specific sites, producing poisonous substances that aid in defense against predators and prevent bacterial, fungal or

viral infections [7]. In the granular layer glands it is possible to find a contractile region, which helps to eject the substances produced in the gland [9]. The substances produced are of various types, and a single species is able to produce a multitude of granular gland substances [10,11]. Among the bioactive molecules produced in granular glands, the main ones are aromatic heterocyclic alkaloids and steroidal substances, guanidinic derivatives, proteins and peptides [10-13]. Among these, peptides were shown to be potential vasodilator agents, analgesics [14], antimicrobial agents [15] and enzyme inhibitors [16]. The AMPs are synthesized as part of the immune response of amphibians to pathogens and continuously released after contraction of myoepithelial cells surrounding the granular glands [9]. In amphibians the antimicrobial peptide precursor contains a signal sequence, an acidic pro-region and a C-terminal domain that contains the sequence of the mature peptide. Before releasing the active AMP three fundamental processes occur: 1) the signal sequence directs the pre-peptide to a specific region of the granular gland, where it remains inactive; 2) then the first proteolytic cleavage sequence occurs, where the signal is removed and the acidic sequence is exposed; 3) when the animal receives a stimulus, i.e. the secretory activity is stimulated, the second proteolytic action occurs, where the region containing the acidic sequence releases the mature peptide [10].

AMP families

Phylloseptins: Phylloseptins are a family of linear, cationic peptides, with 19 – 20 amino acid residues in length (Table 1), which were first isolated from the skin secretion of *Phyllomedusa hypochondrialis* by Leite et al. [11]. These peptides are characterized by the presence of a C-terminal amidation, as well as the highly conserved sequence FLSLI[L]P at the N-terminal [11]. It is also known that phylloseptins are Lys/His-rich peptides, presenting 1 to 3 His residues that may be responsible for net charge variations along the molecule [12]. Structural studies involving circular dichroism (CD) and nuclear magnetic resonance (NMR) experiments revealed that phylloseptins adopt a random coil conformation in water. On the other hand, in the presence of trifluoroethanol (TFE) or anionic vesicles these peptides clearly adopt a-helical conformation stabilized by electrostatic, hydrophobic and capping interactions [13]. Although the mechanisms of action of these molecules still remain unknown, their behavior in anionic environments suggests peptide/membrane adsorption, which would cause membrane collapse and, consequently, cell death [12,13]. Furthermore, phylloseptins have been shown to present broad-spectrum activities against Gram-negative and –positive bacteria [11], fungi [13] and protozoans [12].

Peptide families	Sequences	Theoretical MM (Da)	Theoretical PI	References
Phylloseptin				
PS-1	FLSLIPHAINAVSAIAKHN-NH_2	2267.6	8.76	[12]
PS-2	FLSLIPHAINAVSTLVHHF-NH_2	2367.7	7.02	[12]
PS-3	FLSLIPHAINAVSALANHG-NH_2	2196.5	6.92	[12]
PS-4	FLSLIPHAINAVSTLVHHSG-NH_2	2364.6	7.02	[12]
PS-5	FLSLIPHAINAVSAIAKHS-NH_2	2240.5	8.76	[12]
PS-6	SLIPHAINAVSAIAKHF-NH_2	2040.3	8.51	[12]
PLS-S2	FLSLIPHIVSGVASLAKHF-NH_2	2287.6	8.77	[13]
PLS-S3	FLSLIPHIVSGVASLAIHF-NH_2	2272.6	7.02	[13]
PLS-S4	FLSMIPHIVSGVAALAKHL-NH_2	2255.7	8.77	[13]
PLS-S5	LLGMIPVAISAISALSKL-NH_2	2048.5	8.76	[13]
PLS-S6	FLSLIPHIVSGVASIAKHL-NH_2	2253.6	8.77	[13]
Nigrocin				
Nigrocin-1	GLLSGILGAGKHVVCGLSGLC	1954.3	8.07	[15]
Nigrocin-2	GLLGKILGVGKHIVCGLSGLC	2021.4	9.31	[15]
Nigrocin-OG12	GPLSGILGAGKHIVCGLSGLC	1952.3	8.07	[15]
Nigrocin-OG20	GLLSGVLGVGKKVLCGLSGLC	1973.4	8.90	[15]
Nigrocin-2GRa	GLLSGILGAGKHIVCGLSGLC	1968.4	8.07	[15]
Nigrocin-2GRb	GLFGKILGVGKKVLCGLSGMC	2080.6	9.39	[15]
Nigrocin-2GRc	GLLSGILGAGKNIVCGLSGLC	1945.3	8.06	[15]
Nigrocin-2LVa	GLLSKVLGVGKKVLCGVSGLC	2030.5	9.39	[19]

Nigrocin-2LVb	GILSGILGMGKKLVCGLSGLC	2019.5	8.90	[19]
Nigrocin-2SCa	GILSGILGAGKSLVCGLSGLC	1918.3	8.06	[19]
Nigrocin-2SCb	GILSGVLGMGKKIVCGLSGLC	2005.5	8.90	[19]
Nigrocin-2SCc	GILSNVLGMGKKIVCGLSGLC	2062.5	8.90	[19]
Nigrocin-2VB	SILSGNFGVGKKIVCGLSGLC	2052.4	8.89	[19]
Nigrocin-2HJ	GLLSKVLGVGKKVLCGVSGLC	2030.5	9.39	[19]
Japonicin				
Japonicin-1	FFPIGVFCKIFKTC	1650.0	8.90	[21]
Japonicin-2	FGLPMLSILPKALCILLKRKC	2358.0	9.85	[21]
Japonicin-1CDYa	FFPLALLCKVFKKC			[23]
Japonicin-2CHa	FVLPLLGILPKELCIVLKKNC	2354.0	8.86	[22]
Japonicin-2CHb	VVPAFVLLKKAICIMLKRNC	2259.9	9.85	[22]
Japonicin-2CHc	VVPAFVLLRKAICIMLKRNC	2287.9	10.11	[22]
Japonicin-2CHd	VVPAFVLLKKAICIMFKRNC	2293.9	9.85	[22]
Palustrin				
Palustrin-1	ALFSILRGLKKLGKMGQAFVNCEIYKKC	3159.9	9.79	[24]
Palustrin-2	GFLSTVKNLATNVAGTVLDTIRCKVTGGCRP	3192.7	9.50	[24]
Palustrin-3	GIFPKIIGKGIKTGIVNGIKSLVKGVGMKVFKAGLN NIGNTGCNEDEC	4933.8	9.45	[24]
Palustrin-OG1	GLWDTIKQAGKKFFLNVLDKIRCKVAGGCRT	3467.1	9.90	[24]
Palustrin-OG2	KKFFLKVLTKIRCKVAGGCRT	2397.0	10.59	[27]
Palustrin-2CG1	GLWNTIKEAGKKFAINYLDKIRCGIAGGCKT			[25]
Palustrin-2Cha	GLLSTFKNLATNVAGTVIDTLKCKVTGGCRT	3182.7	9.39	[28]
Palustrin-2ISa	GFMDTAKNVAKNVAVTLLDKLKCKITGGC	3039.6	9.24	[29]
Palustrin-2ISb	GLWNSIKIAGKKLFVNVLDKIRCKVAGGCKTSPDV E	3888.6	9.51	[29]
Parkerin	GWANTLKNVAGGLCKITGAA	1945.2	9.31	[30]
Jingdongin				
Jingdongin-1	FLPLFLPKIICVITKKC	1976.6	9.39	[33]
Jingdongin-2	FLPIVENCSLVCWENNQKC	2239.6	4.59	[33]
Medusin				
Medusin-AC	LLGMIPLAISAISALSKL	1811.3	8.75	[34]
Medusin-PH	LLGMIPVAISAISALSKL	1797.2	8.75	[34]
Medusin-PD	LLGMIPLAISAISSLSKL	1827.3	8.75	[34]
Limnonectin				
Limnonectin-1Fa	SFPFFPPGICKRLKRC	1896.3	10.11	[35]
Limnonectin-1Fb	SFHVFPPWMCKSLKKC	1938.4	9.39	[35]

Hylaranin				
Hylaranin-1	GVLSAFKNALPGIMKIIV-NH_2	1871.3	10.00	[36]
Hylaranin-2	GVLSVIKNALPGIMRFIA-NH_2	1899.3	11.00	[36]
MM: Molecular mass; PI: isoelectric point; Underlined: Sequence fragments containing two cysteine residues responsible to form intermolecular disulfide bonds at the C-terminal region (Rana Box).				

Table 1: Primary sequence, theoretical molecular masses and isoelectric points of amphibian AMP families.

Peptides	**PS-1**	**PS-2**	**PS-3**	**PLS-S1**	**PLS-S2**	**PLS-S3**	**PLS-S4**	**PLS-S5**	**Nigrocin-1**	**Nigrocin-2**
References	[11]	[11]	[11]	[12]	[12]	[12]	[12]	[12]	[12]	[12]
Microorganisms										
Acinetobacter baumannii	-	-	-	6.25	6.25	-	6.25	-	-	-
Acinetobacter calcoaceticus	7.9	3.7	4.1	-	-	-	-	-	-	-
Candida albicans	7.9	15.1	8.2	-	-	-	-	-	51.2	49.5
Enterococcus faecalis	4	-	-	-	-	-	-	-	-	-
Escherichia coli	7.9	-	-	-	-	-	-	-	-	-
Klebsiella pneumonia	-	-	-	-	-	-	-	-	5.1	4.9
Micrococcus luteus	-	-	-	-	-	-	-	-	1.3	1.2
Proteus mirabilis	-	-	-	-	-	-	-	-	>100	>100
Pseudomonas aeruginosa	4	-	-	-	-	-	-	-	38.4	49.5
Saccharomyces cerevisiae	-	-	-	12.5	6.25	-	12.5	-	-	-
Salmonella typhimurium	-	-	-	-	-	-	-	-	11.5	11.1
Serratia marcescens	-	-	-	-	-	-	-	-	>100	>100
Shigella dysenteriae	-	-	-	-	-	-	-	-	5.1	4.9
Staphylococcus aureus	7.9	-	-	6.25	6.25	-	6.25	25	-	-
Streptococcus agalactiae	3.9	1.9	4.1	-	-	-	-	-	-	-
Streptococcus pyogenes	-	-	-	3.12	1.56	12.5	3.12	-	-	-
MIC: Minimum Inhibitory Concentration.										

Table 2. Antimicrobial activities (MIC (μM)) of phylloseptins and nigrocins isolated from *P. hypochondrialis, P. sauvagii* and *P. nigromaculatus*.

Leite et al. [11] isolated six phylloseptins, named PS-1 to PS-6 (Table 1) and tested their antimicrobial and anti-protozoa activities. When tested against *Staphylococcus aureus, Enterococcus faecalis, Escherichia coli and Pseudomonas aeruginosa* (Table 2), PS-1 presented remarkably low minimum inhibitory concentration (MIC) values even when compared with conventional antibiotics such as ampicillin and chloramphenicol [11].

In the same report, atomic force microscopy revealed that PS-1 was able to induce several bubble-like structures on the membrane of *P. aeruginosa*, leading to changes in bacterial morphology [11]. Moreover, PS-4 and PS-5 showed anti protozoa activities against *Trypanosoma cruzi* at low concentration levels [11].

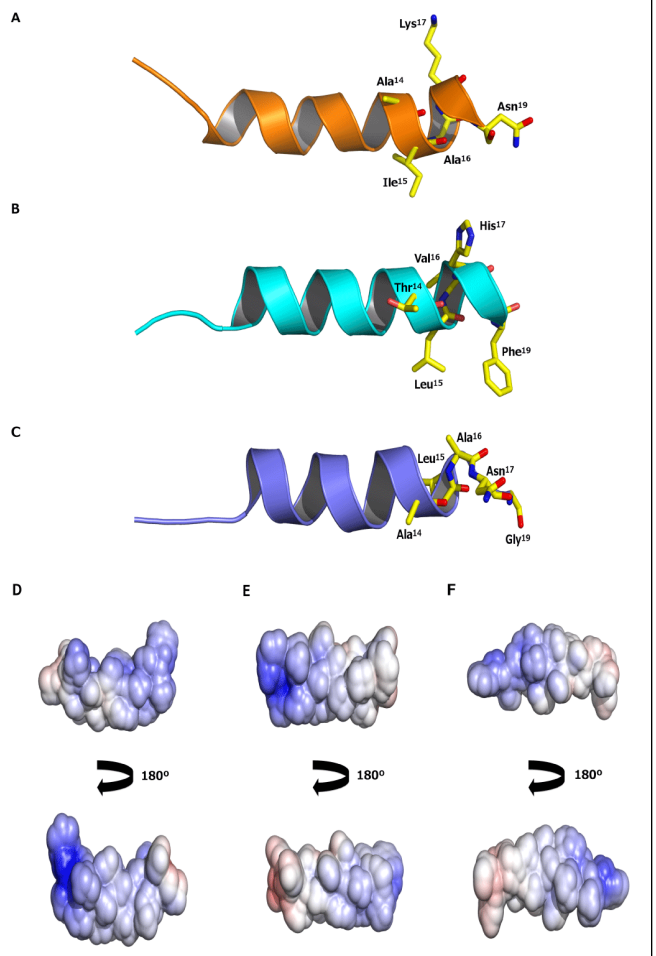

Figure 1: Three-dimensional structures of (A) phylloseptin-1 (pdb code: 2JQ0), (B) phylloseptin-2 (pdb code: 2JPY) and (C) phylloseptin-3 (pdb code: 2JQ1) in the presence of POPC/POPS (3:1) vesicles, resolved by Nuclear Magnetic Resonance (NMR). In sticks (yellow) the divergent residues (positions 14-17 and 19) between the phylloseptins PS-1 (Ala14, Ile15, Ala16, Lys17 and Asn19); PS-2 (Thr14, Leu15, Val16, His17 and Phe19); PS-3 (Ala14, Leu15, Ala16, Asn17 and Gly19) are highlighted. Electrostatic potential of PS-1 (D), -2 (E) and -3 (F) are presented in blue in blue: cationic surface; in red: anionic surface; in white: hydrophobic surface.

Resende et al. [13] reported that PS-1 (Figure 1A), -2 (Figure 1B) and -3 (Figure 1C) also showed remarkable antibacterial (*Acinetobacter calcoaceticus* and *Streptococcus agalactiae*) and antifungal (*Candida albicans*) activities (Table 2) even when compared to some antibiotics such as amoxicillin and tetracyclin [13]. These three peptides present 74% sequence homology and charges of (+1), (0 to +1) and neutral for PS-1 (Figure 1D), PS-2 (Figure 1E) and PS-3 (Figure 1F), respectively. PS-1, -2 and -3 are the only phylloseptins here cited which have their tridimensional structures resolved by Nuclear Magnetic Resonance (NMR) and deposited in the Protein Data Bank (PDB). In addition, more recently, Raja et al. [12] identified five novel phylloseptins, named PLS-S2, -S3, -S4, -S5 and –S6 (Table 1), and also a phylloseptin-1 (PLS-S1) previously reported, using transcriptomic and peptidomic analysis of the skin secretion from *Phyllomedusa sauvagii*. Antimicrobial assays showed that PLS-S1, -S2, -S3 and -S4 were able to eliminate Streptococcus pyogenes colonies completely at low concentrations. PLS-S1, -S2, -S4 and –S5 also revealed potential activities against S. aureus. When tested against Gram-negative bacteria the best MICs were observed for PLS-S1, -S2 and –S4 against *Acinetobacter baumannii* (Table 2). Antifungal activities were also observed for PLS-S1, -S2 and –S4 against *Saccharomyces cerevisiae* (Table 2) [12]. The broad-spectrum activities of phylloseptins against bacteria and fungi in association with their low hemolytic potential make them very attractive for pharmaceutical purposes involving drug design.

Nigrocin

The antimicrobial peptides nigrocin-1 and -2 were first isolated from the Korean frog, *Rana nigromaculata* (now *Pelophylax nigromaculatus*). These peptides, with 21 amino acid residues (Table 1) [14], are lysine-rich, also presenting two conserved cysteines linked by a disulfide bond in the C-terminal region (Rana box), which is characteristic of Ranidae AMPs [15]. Nigrocin-1 shows about 73% of sequence similarity with brevinin-2, while sequence homology between nigrocin-2 and other peptides from Ranidae are low [15,16]. Nigrocin-2 related peptides were also isolated from odorous frog (*Odorrana grahami*) skin secretion and were named nigrocin-2GRa-c [17], and nigrocin-OG12 and -20 (Table 1) [14]. Other homologues have been identified in different frog species: nigrocin-2LVa and -b (*Odorrana livida*), nigrocin-2SCa, -b and -c (*Odorrana schmackeri*), nigrocin-2VB (*Odorrana versabilis*) and nigrocin-2HJ (*Odorrana hejiangensis*) (Table 1) [18].

Unlike other short peptides in this family, nigrocin-2 has no proline residues, presenting different primary structural characteristics from others [15,16,18]. In studies performed by Park et al. [15], despite their different primary structure, nigrocin-1 and -2 showed similar activities against Gram-positive and negative bacteria and also fungi (Table 2), without being highly hemolytic [19]. This probably occurs due to the fact that the polar residues are well interspersed among the hydrophobic residues, interrupting the contiguity of hydrophobicity, which gives the potential to form an amphipathic helix, where leucine and valine residues are aligned on a portion of the helical cylinder, whereas it occupies the remaining surface, like other antimicrobial Ranidae peptides, such as brevinins-1E and ranalexin. However, this peptide is capable of changing from random-coil to amphipathic a-helix depending on the environment (aqueous solution or anionic environment) [17]. This conformational transition could reflect its potential for interaction with membranes, ultimately leading to disruption of cell membrane integrity, indicating that nigrocins could be a good candidate for a new antibiotic agent [20].

Japonicin

The japonicin-1 and -2 peptides (Table 1) were first extracted from the granular glands of *Rana japonica* by Isaacson et al. [21] and show little structural similarity to peptides previously isolated from other Ranidae. They are classified as belonging to novel families designated japonicin-1 and japonicin-2, an extension of the temporin family, but lacking detectable antimicrobial activity. Japonicin-2 was the first amphibian antimicrobial peptide to be identified that contains a disulfide bonded cyclic octapeptide region formed between Cys14 and Cys21. In the case of japonicin-1, a cyclic heptapeptide region occurs,

also known as Rana box (Table 1), which is stabilized by a single disulfide bond involving Cys8 and Cys14 [21].

The antibacterial activities of japonicin have been reported, where both japonicin-1 and -2 were capable of inhibiting the development of Gram-positive and negative bacteria (Table 3) [6,21,22]. In addition, both peptides presented low hemolytic activity.

Peptides	Japonicin-1	Japonicin-2	Japonicin-2CHa	Japonicin-1CDYa	Palustrin-1	Palustrin-2	Palustrin-3	Palustrin-OG1	Palustrin-OG2	Palustrin-2CG1	Palustrin-2Cha	Palustrin-2lSa	Palustrin-2lSb
References	[21]	[21]	[22]	[23]	[14]	[14]	[19]	[14]	[27]	[25]	[28]	[29]	[29]
Microorganisms													
Bacillus licheniformis	-	-	-	-	-	-	-	-	-	19	-	-	-
Candida albicans	-	-	-	-	>100	>100	-	-	-	-	>80	-	-
Enterococcus faecalis	-	-	-	-	-	-	-	-	-	75	-	100	-
Escherichia coli	30	12	100	25	8	78	1	4.6	6.3	>100	>80	100	6.3
Klebsiella pneumoniae	-	-	-	-	-	-	-	-	-	>100	-	-	-
Psychrobacter faecalis	-	-	-	-	-	-	-	-	-	4.5	-	-	-
Rhodococcus rhodochrous	-	-	-	-	-	-	-	-	-	2.3	-	-	-
Serratia rubidaea	-	-	-	-	-	-	-	-	-	4.6	-	-	-
Slime mold	-	-	-	-	-	-	-	-	-	4.5	-	-	-
Staphylococcus aureus	>100	20	>100	>100	>100	>100	-	4.6	6.3	>100	>80	25	6.3
Staphylococcus carnosus	-	-	-	-	-	-	-	-	-	19	-	-	-
MIC: Minimum Inhibitory Concentration.													

Table 3. Antimicrobial activities (MIC (μM)) of japonicins and palustrins isolated from *R. japonica, R. chaochiaoensis, R. dybowskii, L. palustris, O. grahami, A. chunganensis, L. chiricahuens and O. ishikawae*.

More than 300 μM were needed to achieve 50% lysis of red blood cells [6]. Colon and co-workers [22] characterized japonicin-2CHb, japonicin-2CHc, and japonicin-2CHd (Table 1), isolated from *Rana chaochiaoensis*, and demonstrated that two frog species, *R. chaochiaoensis* and *R. japonica*, have a close phylogenetic relationship. However, the two peptides differ by seven amino acid substitutions and this variation is reflected in their different antimicrobial activities. Japonicin-2Cha (Table 1) showed weaker antimicrobial activity against *S. aureus* and E. coli (Table 3) when compared with japonicin-2. It is proposed that this reduced antimicrobial potential observed in japonicin-2Cha could be a consequence of the reduction in cationicity associated with the amino acid substitutions Ala12→Glu and Lys20→Asn, with a net positive charge of +4 at neutral pH in japonicin-2, when compared to japonicin-2CHa (net charge=+2) [21, 22]. Another AMP named japonicin-1CDYa (Table 1) contains six amino acid substitutions compared with japonicin-1 (Table 1). Antimicrobial activities against Gram-positive (*S. aureus*) and Gram-negative bacteria (*E. coli*) were determined by using a serial microdilution assay, and japonicin-1CDYa was able to inhibit *E. coli* and *S. aureus* growth. Although the Rana-originated antimicrobial peptides show a significant amount of variability and diversity, most of them exhibit a single amino acid sequence pattern at their N-terminus, with phenylalanine or glycine as the first amino acid, while their C-terminus contains the Rana box region [23].

Palustrin

The palustrin antimicrobial peptide family was originally isolated by Brasir et al. [24] from the skin secretions of the North American pickerel frog *Rana palustris* (now named *Lithobates palustris*). Palustrin-1 family has 4 peptides with 27-28 amino acid residues and a disulfide bond in a Rana box region (Table 1). Most peptides of the palustrin-2 family present 31 amino acid residues (Table 1) and a cyclic Rana box domain containing 29 amino acid residues stabilized by a disulfide bond [25,26]. The palustrin-3 family has 2 peptides with 48 amino acid residues (Table 1) and a cyclic Rana box region [27].

Palustrin-1 and -2 have antimicrobial activity against fungi and most Gram-positive and -negative bacteria (Table 3) [14]. However, palustrin-3 (Table 1) is active only against E. coli strains (Table 3) [19].

Palustrin-OG1 (Table 1) is member of the palustrin-2 family found in the skin of *Odorrana grahami* with high activity against *E. coli* and *S. aureus* (Table 3) [14]. However, at a low concentration this peptide induces lysis of human erythrocytes, which limits its application as a therapeutic agent [27]. For this reason, palustrin-OG1 was modified to palustrin-OG2 generated through amino acid deletion and substitutions (Table 1). This new peptide showed higher net positive charge, higher amphiphilicity and lower hydrophobicity than its precursor. Furthermore, OG2 showed lower cytotoxicity and higher antimicrobial activity than OG1 [27].

Palustrin-2CG1 is also a member of the palustrin-2 family, identified in the skin secretion of *Amolops chunganensis* (Table 1), and it shares 67.7% homology with palustrin-OG2 from *O. grahami*, displaying antimicrobial activity against various bacteria and fungi (Table 3), except for Gram-positive bacterium *P. aeruginosa* and the fungus *C. albicans*, presenting low hemolytic activity against human erythrocytes [25]. Another peptide from this family is palustrin-2Cha (Table 1), which was identified in *Lithobates chiricahuens* skin secretion [28], exhibiting potent antimicrobial activity against fungi and most Gram-positive and negative bacteria (Table 3), with no hemolytic activity [14,28].

Iwakoshi et al. [29] isolated another palustrin, named palustrin-2ISb (Table 1) from the endangered anuran species (*Odorrana ishikawae*), which consists of 36 amino acid residues including 7 amino acids at C-terminal involved in the cyclic heptapeptide Rana box domain. This peptide primary structure suggests a close relationship with a peptide extracted from the Chinese odourous frog, *Odorrana grahami*, belonging to the palustrin-2 family, presenting antimicrobial activities against *E. coli* and *S. aureus* (Table 3). In this same study, Iwakoshi and colleagues report a synthetic analog, named palustrin-2ISa, which consists of 29 amino acid residues and a cyclic heptapeptide Rana box domain that showed greater antimicrobial activities against *E. coli, S. aureus*, and *C. albicans* than the native peptide (Table 3) [29].

Parkerin

The parkerin peptides were isolated from the Xizang plateau frog, *Nanorana parkeri*, presenting a single sequence composed of 20 amino acid residues (Table 1), which has not been found in any other frog AMPs [30].

Most AMPs from Ranidae share a conserved disulfide bond in a heptapeptide segment at the C-terminal end [31]. Moreover, a cationic nature and a-helical structure can also be observed. At the N-terminal parkerins also present a highly conserved preproregion, followed by a C-terminal domain [32]. The secondary structure of parkerin peptides was investigated by CD spectroscopy and exhibited mainly a random coil conformation in water. On the other hand, in a membrane-mimetic solvent containing 50% TFE and water in 8 mM SDS, the presence of one positive band (190 nm) and two negative dichroic bands at 208 and 222 nm were consistent with the a-helix conformation. The a-helix conformation corresponded to 35% of the secondary structure. This kind of structure facilitates pore formation in bacterial membranes.

Parkerins show a broad spectrum of antimicrobial activity. They are effective against Gram-positive (*S. aureus* and *Enterococcus faecium*), negative bacteria (*E. coli, Serratia marcescens, A. baumannii and K. pneumoniae*) and also against the fungus *C. albicans* (Table 4) [30].

Peptides	**Parkerin**	**Jingdongin-1**	**Medusin-AC**	**Medusin-PH**	**Medusin-PD**	**Limnonectin-1Fa**	**Limnonectin -1Fb**	**Hylaranin-1**	**Hylaranin-2**
References	[30]	[33]	[34]	[34]	[34]	[35]	[35]	[36]	[36]
Microorganisms									
Acinetobacter baumannii	37.5	-	-	-	-	-	-	-	-
B. dysenteriae	-	35	-	-	-	-	-	-	-
Bacillus subtilis	-	9.39	-	-	-	-	-	-	-
Candida albicans	75	18.8	17.7	71.2	35	-	-	8.6	4.2
Enterococcus faecium	37.5	-	-	-	-	-	-	-	-
Escherichia coli	>100	-	-	-	-	35	70	34.2	33.7
Klebsiella pneumoniae	>100	-	-	-	-	-	-	-	-
Serratia marcescens	>100	-	-	-	-	-	-	-	-
Staphylococcus aureus	37.5	4.7	4.4	17.8	17.5	-	-	4.3	4.2

Table 4. Antimicrobial activities (MIC (μM)) of parkerin, medusin, limnonectins and hylaranins isolated from *N. parkeri, A. jingdongensis, A. callidryas, P. hypochondrialis, P. dacnicolor*, *L.fujianensis* and *H. latouchii*. **MIC:**Minimum Inhibitory Concentration.

The killing kinetics was examined by using the colony counting assay. In addition to inhibiting the growth of several microorganisms, parkerin presented no hemolytic activity in a concentration of up to 80 $mg.ml^{-1}$. These results demonstrate that parkerin peptides are good candidates for designing effective antibiotics.

Jingdongin

Jingdongins are a new AMP family isolated from skin secretions of *Amolops jingdongensis* by He et al. [33]. These peptides can be divided into two families, named jingdongin-1, a cationic peptide (net charge +3), and jingdongin-2, an anionic peptide (Table 1). Jingdongin-2 presented two acidic and one basic amino acid residue, which provide a net negative charge to the molecule. On the other hand, jingdongin-1 presents a net positive charge (+3) due to the presence of three Lys residues. Despite their unique primary sequences, jingdongin-1 and -2 present a segment containing a disulfide bond, named Rana box, involving seven amino acid residues; this has also been reported in other AMPs extracted from frogs.

In order to evaluate the antimicrobial activity of these peptides, He and colleagues [33] tested synthetic jingdongin-1 and -2 against different strains of Gram-negative and –positive bacteria and also fungi. The results revealed that jingdongin-1 was able to eliminate colonies of *B. dysenteriae, S. aureus, Bacillus subtilis* and *C. albicans* completely with minimum inhibitory concentration (MIC) values (Table 4) very similar to those from other well-known AMPs from frogs, such as brevinins and temporins [33]. However, no activity was observed for jingdongin-2 against the microorganisms tested, revealing that this peptide cannot be considered an AMP. Furthermore, both jingdongin-1 and -2 were also tested against rabbit red blood cells. It was observed that both peptides presented low hemolytic activity when compared to other AMPs [33]. In conclusion, more studies involving these peptides should be performed in order to better understand the mechanisms of action of jingdongin-1 as a potential AMP, and also to elucidate the possible biological roles of jingdongin-2 [33].

Medusins

Xi et al. [34] reported three highly conserved representatives of a novel antimicrobial peptide family, from skin secretions of three phyllomedusine leaf frog species, *Agalychnis callidryas, P. hypochondrialis* and *Pachymedusa dacnicolor*. These peptides were named medusins and designated as medusins-AC, medusins-PH and medusins-PD (Table 1) [34].

Medusins are close relatives of phylloseptins, with a fully conserved N-terminal hexapeptide sequence and a similar C-terminal tetrapeptide amide sequence. However, phylloseptins have a His residue at position 7 from the N-terminus and in the penultimate position of the C-terminus. Since the medusin antimicrobial peptide family is novel and its structure is similar to that of phylloseptins, maybe some previous data should be reviewed. For example, phylloseptin-S5 isolated from *Phyllomedusa sauvagei* skin was classified when medusins were still unknown. However, phylloseptin-S5 and medusin-PD show an identical primary structure (Table 1). For this reason, Xi and colleagues [34] suggested that phylloseptins-S5 peptide be renamed medusin-PS.

Medusins consist of 18 amino acids with a highly conserved sequence (Table 1). Their primary structure contains approximately 72% of hydrophobic residues, a single Lys residue, 22% neutral-polar residues and a C-terminal amide. Furthermore, it has a post-translational modification that serves two purposes: 1) the canonical protein negatively-charged C-terminal carboxyl group is neutralized, thus increasing the net positive charge of the peptide; and 2) the more hydrophobic nature of the carboxyamide facilitates membrane interaction. This group of peptides exhibited deleterious activities against the Gram-positive bacterium *S. aureus* and the yeast *C. albicans* (Table 4), and also presented hemolytic activity at high concentrations [34].

Limnonectin

The limnonectin peptide [35] was first identified in a cDNA library from the skin secretion of a species of Chinese frog and Fujian large-headed frog, Limnonectes fujianensis. They discovered two 16-mer peptides of novel primary structures, named limnonectin-1Fa and limnonectin-1Fb (Table 1).

Structurally, limnonectins present 16 amino acid residues with a possible disulfide bond between Cys10 and Cys16. The C-terminal disulfide bonded loops of the limnonectins contain five amino acid residues, among them basic Arg or Lys. A similar structure was found in brevenins, esculentins and ranatuerins, which are all antimicrobial peptides isolated from Ranid frogs. The standard arrangement of disulfide bonds provides the structure with enhanced stability, and the group of these features has the potential to provide valuable insights for the design of more effective therapeutics [35].

Limnonectin-1Fa and -1Fb displayed a highly specific activity against the Gram-negative bacterium E. coli, demonstrating a low effective MIC value (Table 4). However, both were found to be ineffective against the Gram-positive bacterium *S. aureus*, as well as the pathogenic yeast *C. albicans* (Table 4). In addition, none of the peptides demonstrated significant hemolytic activities [35].

Hylaranins

More recently, two novel peptides, named hylaranin-L1 and hylaranin-L2, belonging to a new family of amphibian antimicrobial peptides, were isolated [36] from *Hylarana latouchii* skin secretion. Both hylaranins are cationic peptides, with 18 amino acid residues and amidated C-termini (Table 1). Despite their high identities, these peptides differ in their primary sequence at positions 5, 6, 15, 16 and 18 (Ala5, Phe6, Lys15, Ile16 and Val18 for hylaranin-L1; Val5, Ile6, Arg15, Phe16 and Ala18 for Hylaranin-L2). Bioinformatic techniques have shown that their primary structure presents no similarity with any antimicrobial peptide primary sequence from frogs or any other organism studied so far. In addition, hylaranin-L1 and –L2 secondary structures were also predicted, revealing that both peptides present two a-helical domains interrupted by a prolyl residue [36]. Moreover, helical wheel models also showed that both peptides have an amphipathic character with hydrophobic residues occurring on one side of the molecule and cationic residues (Lys7 and Lys15 in hylaranin-L1; Lys7 and Arg15 in hylaranin-L2) on the opposite side.

Lin and colleagues [36] performed antimicrobial assays using both hylaranin-L1 and –L2, comparing their activities with those of ampicillin and the well-known AMP melittin. These peptides were able to completely eliminate *E. coli* colonies, at higher concentrations (Table 4) when compared to ampicillin and melittin. However, hylaranins presented high antimicrobial activities against *S. aureus* and *C. albicans* (Table 4) with lower hemolytic activities against horse erythrocytes in comparison to melittin [36]. These data revealed that

hylaranins are a promising new family of AMPs, which was effective against Gram-positive bacteria and fungi without presenting high hemolytic activities, making them a good target for future studies.

Prospects for Development of New Anti-Infective Therapeutic Drugs and Industrial Applications

Over recent years contamination by multiple antibiotic-resistant pathogenic microorganisms has emerged and became a major health concern. This situation has led to an intensive search for more effective molecules against these pathogens [36]. The first people to use natural resources through amphibian secretion in order to obtain relief or cure for diseases were indigenous, who also used these resources for feeding and protection [3,37,38].

Among the peptides obtained from amphibian secretions, magainin has received most attention in the pharmaceutical area. Magainin is a cationic peptide extracted from the secretions of *Xenopus laevis*, with a broad spectrum of action against pathogens, through its interaction with anionic phospholipids in the microbial cell membrane, leading to its disruption [39,40].

In 1999, the antibacterial drug called Pexiganan® was submitted for approval by the Food and Drug Administration (FDA). This drug, developed by the biotechnology company Dipexium Pharmaceuticals, was derived from magainin type II, an AMP with 22 amino acid residues (GIGKFLKKAKKFGKAFVKILKK-NH_2). The molecule is synthesized as a single diastereomer with all chiral centers of pre-defined stereochemistry [38,40].

Pexiganan shows antimicrobial activities against Gram-positive bacteria such as methycillin-resistant *S. aureus* (MRSA) and Gram-negative microorganisms. It is effective in topical treatment of wounds, and is indicated for treating "diabetic ulcers" and "diabetic foot infections" [38]. Pexiganan acts by causing bacterial cell lysis as well as being an effective inhibitor of bacterial proliferation, including for resistant bacteria [38,40]. Additionally, it is known that Pexiganan is generally effective against a broad range of pathogens including yeasts, filamentous fungi, viruses and protozoa in concentrations which do not harm normal mammalian cells [41].

However, given the complexity of the research in this area, few antibiotic peptides are at an advanced preclinical phase [38]. Because of that, there is still no amphibian peptide approved by the Food and Drug Administration (FDA). Among the obstacles to be overcome to allow the proper use of AMPs as therapeutic drugs is the instability of the orally administered peptide due to the action of proteolytic digestive enzymes. In addition, the gastrointestinal tract may not be able to adsorb the AMP [38]. Another problem is the chemical instability of the formulations during long-term storage. Over time, the peptides can degrade via a number of mechanisms including deamination, oxidation, hydrolysis, disruption of disulfide bonds and racemization [37,38]. Moreover, the high flexibility of the peptide chain can lead to high rates of deamination. Highly self-associating peptides may even form micelles, affecting their ability to cross membranes [38,42].

An alternative strategy for overcoming problems related to AMP degradation after oral administration may be the use of nanotechnology, by protecting the peptides within a polymeric structure that would direct them to appropriate sites of infections [43]. Studies have shown that magainin covalently bound to insoluble polymeric spheres can still prevent bacterial growth [37]. This technique could provide protection and enhance the pharmacokinetics of easily degradable AMPs, as well as reduce the rejection and tissue damage [43].

Current studies continue to bring new expectations about the viability of AMPs extracted from secretions of frogs in order to obtain new classes of drugs, since AMPs are active against a broad spectrum of pathogens (bacteria, yeasts, filamentous fungi, viruses and protozoa) in concentrations not harmful to normal mammalian cells. AMPs usually have a short sequence of amino acid residues, which may allow production of synthetic analogues through changes in their primary sequence length, charge and hydrophobicity without compromising the pharmacological action of the peptides [41].

In summary, although none of the classes described in this review has yet been developed as drugs by pharmaceutical companies, the amphibian skin should be further studied as a rich source of biomolecules with remarkable biotechnological potential for developing bioproducts with pharmacological potential. Furthermore, the discovery of peptides mentioned in this review, as well as novel and unusual classes, could be used as models to contribute in the design of antimicrobial products that are more effective than the drugs already on the market.

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