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Antimicrobial peptides from frog skin: biodiversity and therapeutic promises

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1. ABSTRACT

More than a thousand antimicrobial peptides (AMPs) have been reported in the last decades arising from the skin secretion of amphibian species. Generally, each frog species can express its own repertoire of AMPs (typically, 10–20 peptides) with differing sequences, sizes, and spectrum of action, which implies very rapid divergence, even between closely related species. Frog skin AMPs are highly potent against antibiotic-resistant bacteria, protozoa, yeasts, and fungi by permeating and destroying their plasma membrane and/or inactivating intracellular targets. These peptides have attracted considerable interest as a therapeutic alternative to conventional anti-infective agents. However, efforts to obtain a new generation of drugs using these peptides are still challenging because of high associated R&D costs due to their large size (up to 46 residues) and cytotoxicity. This review deals with the biodiversity of frog skin AMPs and assesses the therapeutic possibilities of temporins, the shortest AMPs found in the frog skin, with 8-17 residues. Such short sequences are easily amenable to optimization of the structure and to solution-phase synthesis that offer reduced costs over solid-phase chemistry.

2. INTRODUCTION

The dermatous exocrine glands of many species of Anura (frogs and toads) synthesize enormous quantities of a rich variety of hormones and neuropeptides that belong to families that have their counterparts in the brain and the gut of mammals, i.e. the so-called brain-skin-gut triangle (1). Examples are many and include thyrotropin-releasing hormone, bradykinins, angiotensins, tachykinins, bombesin/gastrin-releasing peptide, hypophysiotropic neuropeptides, prokineticin, sauvagine, calcitonin gene-related peptide, pancreatic polypeptide/peptide tyrosine-tyrosine/neuropeptide tyrosine, xenopsin, crinia-angiotensin, and the D-amino acid containing opioids, dermorphin and deltorphins (2, 3). Glands may release their content onto the skin surface by a holocrine mechanism involving the rupture of the plasma membrane and the extrusion of the secretory granules through a duct opening to the surface (4-6). Serous glands of Anuran also produce a rich arsenal of gene-encoded antimicrobial peptides (AMPs) thought to be involved in the defense of the naked skin against noxious microorganisms and to aid in wound repair (7-10). These peptides rapidly kill a broad range of bacteria, yeasts, fungi and protozoa by permeating and destroying their plasma membrane and/or inactivating intracellular target (11-14). This prevents a target organism from developing resistance to the peptide. Hence, these peptides have attracted considerable interest as a possible new generation of antibiotics, especially for antibiotic-resistant pathogens.

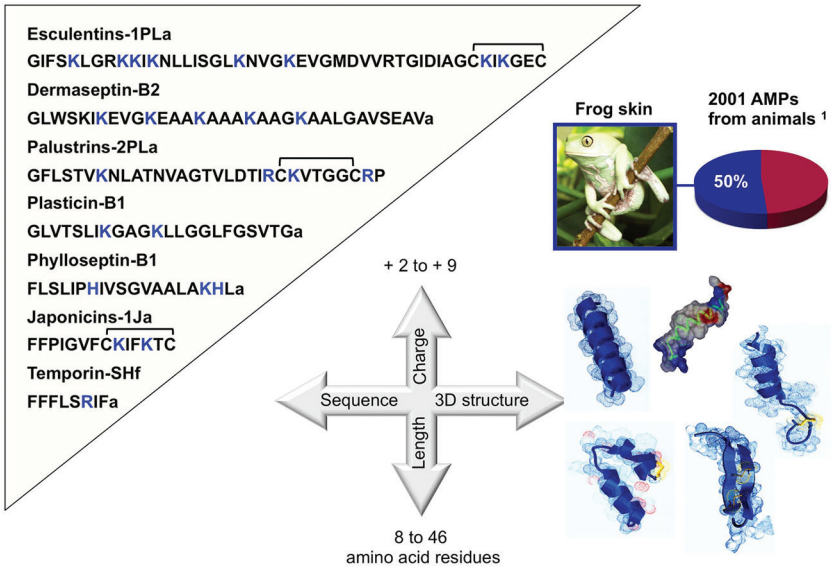

Figure 1. Skin AMPs from Anurans are a heterogeneous class of peptides. They differ in size (from 8 to 46 residues), amino acid sequence, molecular charge, and tridimensional structure. Basic residues are indicated in blue in the sequence of the AMPs. Lines between C residues represent intrachain disulfide bridges.[1]: Data from The Antimicrobial Peptide Database (http://aps.unmc.edu/AP/main.php). Amphibian AMPs account for 50% of the animal peptides contained in the database (last update: January 29, 2016).

Frog skin AMPs are an extremely heterogeneous class of peptides, having only three common feature, which are considered to be a prerequisite for their lytic activity: (i) a net positive charge (due to the presence of basic amino acids), (ii) the presence of at least 50% hydrophobic amino acids, and (iii) a propensity to form amphipathic alpha helix and/or beta sheet structures upon their interaction with the phospholipid membrane of the target cell. Besides that, they differ dramatically in size (from 8 to 46 residues) and amino acid sequence, and do not share any signature pattern or conserved motif responsible for cytolytic activity (Figure 1).

3. BIODIVERSITY OF ANTIMICROBIAL PEPTIDES FROM FROG SKIN

Frog skin AMPs have been characterized across fourteen frog families belonging to both Archaeobatrachia and Neobatrachia (Table 1). The nature and number of these AMPs in each anuran genus/species is given in the Table 1 on the basis of the Amphibian AMPs listed in the Antimicrobial Peptide Database (http://aps.unmc.edu/AP/main.php). The names of the frog and toad families, genera and species were obtained from the updated classification of anurans found in Amphibian Species of the World (http://research.amnh.org/vz/herpetology/amphibia/).). Even though the Table 1 gives a good overview of the peptide diversity found in the frog skin, it is certainly underestimated because many peptides described in the literature are not listed in APD. As indicated in Table 2, anuran AMPs are divided into more than 40 major peptide families on the basis of sequence similarities. The names of the peptides in Table 2 follow the recent proposed guidelines for antimicrobial peptide nomenclature (15-18). In addition, a number of orphan peptides, which do not resemble any members of the other peptide families, have been described. For a complete list of all known frog skin AMPs see also the UniProt Knowledgebase(http://www.uniprot.org/uniprot/).

3.1. Intraspecies diversity of frog skin AMPs: each species of frog has a customized arsenal of peptide weapons

As a rule, frog species in which skin AMPs have been found typically secrete a custom repertoire of 10-20 antimicrobial peptides of differing sizes, sequences, charges, hydrophobicity, tridimensional structures and spectrum of action, which belong to different AMP families. For instance, the skin secretions of the South American waxy monkey tree frog *Phyllomedusa sauvagii* (family: Hylidae, sub-family: phyllomedusinae) contains 21 distinct AMPs that may be grouped into six known peptide families on the basis of amino acid sequence similarity (Table 3). These are the dermaseptins (*stricto sensu*), which share a signature pattern consisting of a conserved Trp residue at position 3 and an AA(A/G)KAAL(G/N)A consensus motif in the midregion, phylloseptins, plasticins, which contain 1 to 4 repeats of the glycine-zipper motif GXXXG (where X is any residue), dermatoxins, phylloxins, and the “orphan” peptide dermaseptin S9, an □lpha-helical antimicrobial peptide with a hydrophobic core and cationic termini (9, 19, 20).

The skin secretion of the North American pickerel frog *Lithobates* (*Rana*) *palustris* (family:

Table 1. Distribution, number and nature of AMPs in Anurans

Anuran family[1] (Subfamily) Genus	Number of AMPs[2]	Species (number of AMPs): AMP (APD ID)[3]
Alsodidae Alsodes	5	*A. montanus* (5): Ascaphin-1M (AP01334), -3M (AP01335), -4M (AP01336), -5M (AP01337), -7M (AP01338)
Alytidae Alytes	9	*A. obstetricans* (7): Alyteserin-1a (AP01457), -1b (AP01458), -1c (AP01459), -1d (AP01460), -2a (AP01461), -2b (AP01462), -2c (AP01463)
		A. maurus (2): Alyteserin-2Ma (AP02013), -2Mb (AP02019)
Arthroleptidae (Leptopelinae) Leptopelis	1	*L. aubryi* (formerly *Hyla punctata*) (1): Hylaseptin-P1 (AP01249)
Ascaphidae Ascaphus	8	*A. truei* (8): Ascaphin-1 (AP01216), -2 (AP01217), -3 (AP01218), -4-(AP01219), -5 (AP01220), -6 (AP01221), -7 (AP01222), -8 (AP01223)
Bombinatoridae Bombina	46	*B. maxima* (31): Maximin-1 (AP00058), -2 (AP00059), -3 (AP00060), -4 (AP00061), -5 (AP00062), -6 (AP00063), -7 (AP00064), -8 (AP00065), -9 (AP00552), -15 (AP01730), -28 (AP01731), -31 (AP01732), -32 (AP01733), -39 (AP01734), -41 (AP01735), -42 (AP01741), -45 (AP01736), -49 (AP01738), -63 (AP01739), -68 (AP01740), -77 (AP01742), -78 (AP01743) -H1 (AP00832), -H2 (AP00525), -H3 (AP00526), -H4 (AP00527), -H5 (AP00497), -H7 (AP01744), -H39 (AP01737), -S4 (AP00547), PR-bombesin (AP01233)
		B. orientalis (9): Bombinin-H7 (AP00795), Bombinin-like peptide 1 (AP00050), Bombinin-like peptide 2 (AP00051), Bombinin-like peptide 3 (AP00052), Bombinin-like peptide 4 (AP00053), Bombinin-like peptide 7 (AP00054), Bombinin GH-1L (AP00796), Bombinin GH-1D (AP00797), Feleucin-BO1 (AP02419)
		B. variegata (6): Bombinin (AP00049), Bombinin-H1 (AP00055), -H2 (AP00793), -H3 (AP00794), -H4 (AP00056), -H5 (AP00057)
Bufonidae Bufo	3	*B. gargarizans* (3): Buforin-I (AP00307), -II (AP00308), BG-CATH37 (AP02580)
Ceratophryidae Ceratophrys	1	*C. calcarata* (1): Ceratoxin (AP02123)
Dicroglossidae (Dicroglossinae) Euphlyctis	3	*E. cyanophlyctis* (3): Temporin-ECa (AP02209), Buforin-EC (AP02210), Cyanophlyctin (AP02211)
Hoplobatrachus	4	*H. tigerinus* (formerly *Rana tigerina*) (4): Tigerinin-1 (AP00303), -2 (AP00304), -3 (AP00305), -4 (AP00306)
Limnonectes	11	*L. fragilis* (2): Lf-CATH1 (AP02306), -CATH2 (AP02307)
		L. fujianensis (2): Limnonectin-1Fa (AP01747), -1Fb (AP01748)
		L. kuhlii (7): Ranacyclin-B-LK1 (AP01912), -LK2 (AP01913), Temporin-LK1 (AP02106), Gaegurin-LK1 (AP02107), -LK2 (AP02108), Rugosin-LK1 (AP02109), -LK2 (AP02110)
Nanorana	3	*N. parkeri* (3): Japonicin-1Npa (AP01577), -1Npb (AP01579), Parkerin (AP01583)
Hylidae (Hylinae) Hyla	2	*H. simplex* (2): Hylain-1 (AP02487), -2 (AP02488)
Hypsiboas	16	*H. albopunctatus* (1): Hylin-a1 (AP01331)
		H. lundii (2): Hylin-b1 (AP01332), -b2 (AP01333)
		H. picturatus (formerly *Hylarana picturata*) (8): Brevinin-1PTa (AP01432), -1PTb (AP01433), -2PTa (AP01427), -2PTb (AP01428), -2PTc (AP01429), -2PTd (AP01430), -2PTe (AP01431), Temporin-PTa (AP01434)
		H. pulchellus (3): P1-Hp-1971 (AP02385), P2-Hp-1935 (AP02386), P3-Hp-1891 (AP02387)
		H. raniceps (1): Raniseptin 1 (AP02384)
		H. semilineatus (1): Hs-1 (AP02527)

(Contd...)

Table 1. *(Continued)*

Anuran family[1] (Subfamily) Genus	Number of AMPs[2]	Species (number of AMPs): AMP (APD ID)[3]
Pseudis	4	*P. paradoxa* (4): Pseudin-1 (AP00475), -2 (AP00476), -3 (AP00477), -4 (AP00478)
Sphaenorhynchus	2	*S. lacteus* (2): Frenatin-2.1S (AP02374), -2.2S (AP02375)
Hylidae (Pelopdryadinae) Litoria	72	*L. aurea, L. raniformis* (12): Aurein-1.1 (AP00012), -1.2 (AP00013), -2.1 (AP00014), -2.2 (AP00015), -2.3 (AP00016), -2.4 (AP00017), -2.5 (AP00018), -2.6 (AP00019), -3.1 (AP00020), -3.2 (AP00021), -3.3 (AP00022), -5.2 (AP02005)
		L. caerula (9): Caerin-1.2 (AP00241), -1.3 (AP00242), -1.4 (AP00243), -1.5 (AP00244), -3.2 (AP00254), -3.3 (AP00255), -3.4 (AP00256), -4.1 (AP00257), -4.3 (AP00259)
		L. caerula, hybrid *L. caerulea/L. splendida* (1): Caerin-4.2 (AP00258)
		Hybrid *L. caerulea/L. splendida* (3): Caerin-2.6 (AP02008), -2.7 (AP02009), -1.20 (AP02010)
		L. chloris (2): Caerin-1.8 (AP00247), -1.9 (AP00248)
		L. citropa (6): Citropin-1.1 (AP00351), -1.1.3 (AP02011), -1.2 (AP00352), -1.3 (AP00353), -2.1 (AP00638), -2.1.3 (AP00639)
		L. dahlii (11): Dahlein-1.1 (AP00696), -1.2 (AP00697), -4.1 (AP00698), -4.2 (AP00699), -4.3 (AP00700), -5.1 (AP00701), -5.2 (AP00702), -5.3 (AP00703), -5.4 (AP00704), -5.5 (AP00705), -5.6 (AP00706)
		L. eucnemis (3): Maculatin-1.3 (AP00640), -1.4 (AP00770), Caerin-1.11 (AP00769)
		L. ewingii (1): Uperin-7.1 (AP00327)
		L. fallax (3): Fallaxidin-3.2 (AP01236), -3.1 (AP01237), -4.1 (AP01242)
		L. genimaculata (3): Maculatin-1.2 (AP00261), -3.1 (AP00263), -1.1.1 (AP01270)
		L. genimaculata, L. eucnemis (2): Maculatin-1.1 (AP00260), -2.1 (AP00262)
		L. gilleni (1): Caerin-2.5 (AP00252)
		L. gracilenta (4): Caerin-1.17 (AP01697), -1.18 (AP01653), -1.19 (AP01654), -3.5 (AP01698)
		L. infrafrenata (3): Frenatin-1 (AP02006), -2 (AP02007), -3 (AP00434)
		L. splendida (5): Caerin-1.10 (AP00345), -2.1 (AP00249), -2.2 (AP00250), -2.4 (AP00251), -3.1 (AP00253)
		L. splendida, L. rothii (1): Caerin-1.1 (AP00240)
		L. xanthomera (2): Caerin-1.6 (AP00245), -1.7 (AP00246)
Hylidae (Phyllomedusinae) Agalychnis	19	*A. annae* (5): Dermatoxin-A1 (AP00907), Dermaseptin-A3 (AP00963), -A4 (AP00962, AP00967), DRP-AA-3-4 (AP01384), Plasticin-A1 (AP01383)
		A. callidryas (5): Plasticin-C1 (AP01387), -C2 (AP01388), Medusin-PH (AP02176), -PD (AP02177), Dermaseptin-C3 (AP00965)
		A. callidryas, A. lemur (1): Phylloseptin-L1 (AP00973)
		A. dacnicolor (formerly *Pachymedusa dacnicolor*) (6): Dermatoxin-DA1 (AP00908), Dermaseptin-DA2 (AP00959), -DA3 (AP00968), -DA4 (AP01546), Plasticin-DA1 (AP01385), DRP-PD-3-7 (AP01386)
		A. lemur (1): Dermaseptin-L1 (AP00964)
		A. spurelli (formerly *A. litodryas*) (1): Dermaseptin-LI1 (AP00960)
Phyllomedusa	74	*P. baltea* (1): Balteatide (AP02411)
		P. bicolor (15): Dermaseptin-B1 (AP00293), -B2 (AP00001), -B3 (AP00165), -B4 (AP00163), -B5 (AP00162), -B6 (AP00756), -B7 (AP00936), -B8 (AP00937), -B9 (AP00164), Phylloxin-B1 (AP00167), Phylloseptin-B1 (AP00911), Dermatoxin B1 (AP00315), SPYY (AP00400), Plasticin-B1 (AP00737), -B1a (AP00938)

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Table 1. *(Continued)*

Anuran family[1] (Subfamily) Genus	Number of AMPs[2]	Species (number of AMPs): AMP (APD ID)[3]
		P. distincta (6): Distinctin (AP00493), Dermaseptin-DI1 (AP00966), -DI2 (AP00958), -DI3 (AP00957), -DI4 (AP00961), -DI5 (AP00969)
		P. hypochondrialis (30): Phylloseptin-H2 (AP00757), -H3 (AP00758), -H4 (AP00761), -H5 (AP00762), -H7 (AP00974), -H8 (AP00975), -H9 (AP00976), -H12 (AP00971), -H13 (AP00970), -O1 (AP00759), -O2 (AP00760), Dermaseptin DPh-1 (AP00763), -H1 (AP00942), -H2 (AP00943), -H3 (AP00944), -H4 (AP00945), -H5 (AP00946), -H6 (AP00947), -H7 (AP00948), -H8 (AP00949), -H9 (AP00950), -H11 (AP00972), -H12 (AP00952), -H13 (AP00953), -H15 (AP00955), Hyposin-H1 (AP00902), -H2 (AP00903), -H3 (AP00904), -H4 (AP00905), -H5 (AP00906)
		P. hypochondrialis, P. nordestina (1): Phylloseptin-H6 (AP00954)
		P. hypochondrialis/azurea (3): Phylloseptin-12 (AP01350), Dermaseptin-H4 (AP01351), -H5 (AP01352)
		P. hypochondrialis, P. oreades (1): Dermaseptin-01 (or -H10) (AP01389)
		P. sauvagii (16): Dermaseptin-S1 (AP00157), -S2 (AP00158), -S3 (AP00159), -S4 (AP00160), -S5 (AP00161), -S6 (AP00933), -S7 (AP00934), -S8 (AP00935), -S9 (AP00764), -S11 (AP00939), -S12 (AP00940), -S13 (AP00941), Phylloxin-S1 (AP00901), Dermatoxin-S1 (AP00909), Plasticin-S1 (AP00910), Phylloseptin-1 (AP01581)
		P. tarsius (1): Dermaseptin-TA1 (AP00956)
Hyperoliidae Kassina	8	*K. senegalensis* (4): Galensin (AP00311), Kassinatuerin-1 (AP00556), Kassorin S (AP01746), Senegalin (AP02174)
		K. maculata (4): Kassinatuerin-2Ma (AP01449), -2Mb (AP01450), -2Mc (AP01451), -2Md (AP01452)
Leptodactylidae (Leptodactylinae) Leptodactylus	16	*L. fallax* (1): Ocellatin-F1 (AP00533)
		L. latrans (formerly *Leptodactylus ocellatus*) (6): Ocellatin-1 (AP00543), -2 (AP00544), -3 (AP00545), -4 (AP00894), -5 (AP01541), -6 (AP01542)
		L. pentadactylus (2): Ocellatin-P1 (AP00540), Leptoglycin (AP01405)
		L. pustulatus (3): Ocellatin-PT4 (AP02598), -PT7 (AP02599), -PT8 (AP02600)
		L. syphax (1): Ocellatin-S1 (AP01401)
		L. validus (3): Ocellatin-V1 (AP01402), -V2 (AP01403), -V3 (AP01404)
Myobatrachidae Crinia	4	*C. deserticola* (1): Deserticolin 1 (AP01262)
		C. signifera (1): Signiferin 2.2 (AP01655)
		C. signifera, C. deserticola (1): Signiferin 2.1 (AP01260)
		C. riparia (1): Riparin 2.1 (AP01261)
Pseudophryne	6	*P. guntheri* (6): PG-L (AP00666), -KI (AP00667), -KII (AP00668), -KIII (AP00669), -SPI (AP00670), -SPII (AP00671)
Uperoleia	11	*U. inundata* (11): Uperin-2.1 (AP00316), -2.2 (AP00317), -2.3 (AP00318), -2.4 (AP00319), -2.5 (AP00320), -2.7 (AP00321), -2.8 (AP00322), -3.1 (AP00323), -3.5 (AP00324), -3.6 (AP00325), -4.1 (AP00326)
Pipidae Hymenochirus	5	*H. boettgeri* (5): Hymenochirin-1B (AP01964), -2B (AP01965), -3B (AP01966), -4B (AP01967), -5B (AP01968)
Pseudhymenochirus	11	*P. merlini* (11): Hymenochirin-1Pa (AP02309), -1Pb (AP02310), -5Pa (AP02311), -5Pb (AP02312), -5Pc (AP02313), -5Pd (AP02314), -5Pe (AP02315), -5Pg (AP02316), -5Ph (AP02317), Pseudhymenochirin-1Pb (AP02318), -2Pa (AP02319)

(Contd...)

Table 1. *(Continued)*

Anuran family[1] (Subfamily) Genus	Number of AMPs[2]	Species (number of AMPs): AMP (APD ID)[3]
Xenopus	35	*X. amieti* (4): CPF-AM1 (AP00500), -AM4 (AP01129), Magainin-AM2 (AP01128), PGLa-AM1 (AP01130)
		X. andrei (2): XPF-AN1 (AP02492), PGLa-AN2 (AP02493)
		X. borealis (1): CPF-B1 (AP01622)
		X. clivii (1): CPF-C1 (AP01695)
		X. fraseri (1): CPF-RP-F1 (AP02448)
		X. fraseri, X. andrei (1): Magainin-F3 (AP02449)
		X. laevis (6): Magainin 1 (AP00771), Magainin 2 (AP00144), Peptide PGQ (AP00209), PGLa (AP00210), PGLa-H (AP01814), XPF (AP00239)
		X. lenduensis (2): CPF-L1 (AP01906), -L2 (AP01907)
		X. petersii (4): CPF-P2 (AP01901), -P3 (AP01902), -P4 (AP01903), -P5 (AP01904)
		X. pygmaeus (1): CPF-PG1 (AP01905)
		X. muelleri (6): Magainin-M1 (AP02481), CPF-M1 (AP02482), Magainin-MW1 (AP02483), PGLa-MW1 (AP02484), CPF-MW1 (AP02485), -MW2 (AP02486)
		X. tropicalis (6): XT-1 (AP00424), -2 (AP00425), -4 (AP00426), CPF-ST3 (AP00427), Pxt-2 (AP02596), -5 (AP02597)
Ranidae Amolops	42	*A. afghanus* (4): Nigrocin-AA1 (AP02607), Brevinin-1AA1 (AP02613), -1AA2 (AP02614), Esculentin-1AA1 (AP02617)
		A. chunganensis (11): Temporin-CG1 (AP02054), -CG2 (AP02055), Temporin-CG3 (AP02056), Esculentin-2CG1 (AP02057), Palustrin-2CG1 (AP02058), Brevinin-1CG1 (AP02059), -1CG2 (AP02060), -1CG3 (AP02061), -1CG4 (AP02062), -1CG5 (AP02063), -2CG1 (AP02064)
		A. hainanensis (2): Hainanenin-1 (AP01953), -5 (AP01954)
		A. jinjiangensis ? (*formerly Amolops jingdongensis*) (6): Jindongenin-1a (AP01778), Palustrin-2AJ1 (AP01779), -2AJ2 (AP01780), Brevinin-1-AJ1 (AP02020), Temporin-AJ8 (AP02021), Jingdongin-1 (AP02022)
		A. loloensis (15): Brevinin-ALa (AP00860), -ALb (AP00861), Temporin-ALa (AP00863), -ALd (AP01931), -ALe AP01932), -ALf (AP01933), -ALg (AP01934), -ALh (AP01935), -ALi (AP01936), -ALj (AP01937), -ALk (AP01938), Cathelicidin-AL (AP01898), Ranacyclin-B-AL1 (AP01914), Esculentin-2-ALa (AP01929), -ALb (AP01930)
		A. ricketti (4): Brevinin-1RTa (AP01894), -1RTb (AP01895), -2RTa (AP01896), -2RTb (AP01897)
Babina	15	*B. adenopleura* (1): Brevinin-1AN1 (AP02413)
		B. daunchina (2): Brevinin-1DN1 (AP02414), Ranatuerin-2DN1 (AP02415)
		B. pleuraden (formerly *Rana pleuraden*) (12): Pleurain-A1 (AP00570), -A2 (AP00571), -B1 (AP02262), Pleurain-C1 (AP02263), -D1 (AP02264), -E1 (AP02265), -G1 (AP02266), -J1 (AP02267), -M1 (AP02268), -R1 (AP02269), -N1 (AP02270), -D4 (AP02271)
Clinotarsus	5	*C. curtipes* (5): B1CTcu-1 (AP02564), -2 (AP02565), -3 (AP02566), -4 (AP02567), -5 (AP02568)
Glandirana	9	*G. emeljanov* (formerly *Rana rugosa*, Korea) (6): Gaegurin-1 (AP00085), -2 (AP00086), Gaegurin-3 (AP00087), Esculentin-2EM (AP00088), Brevinin-1EMa (AP00089), -1EMb (AP00090)
		G. rugosa (formerly *Rana rugosa*, Japan) (3): Rugosin-A (AP00091), -B (AP00092), -C (AP00093)
Huia	5	*H. schmackeri* (5): Brevinin-2HS1 (AP00880), -2HS2 (AP00881), -2HS3 (AP00882), -1HS2 (AP00883), Odorranain-P1a (AP01303)

(Contd...)

Table 1. *(Continued)*

Anuran family[1] (Subfamily) Genus	Number of AMPs[2]	Species (number of AMPs): AMP (APD ID)[3]
"Hylarana"	19	"*H." latouchii* (19): Brevinin-1LTa (AP01345), -1LTb (AP01349), Temporin-LTa (AP01346), -LTb (AP01347), -LTc (AP01348), Brevinin-1LTb (AP01349), -1LT1 (AP01445), -1LT2 (AP01446), -2LTa (AP02298), -2LTb (AP02299), -2LTc (AP02300), Esculentin-1LTa (AP02301), -2LTa (AP02302), Palustrin-2LTa (AP02303), Temporin-LT1 (AP01447), -LT2 (AP01448), -LTe (AP02304), Hylaranin-L1 (AP02420), -L2 (AP02421)
Hylarana	20	*H. erythraea* (1): B2RP-ERa (AP01547)
		H. taipehensis (19): Temporin-TP1 (AP02458), -TP2 (AP02459), -TP3 (AP02460), -LF1 (AP02467), Brevinin-1TP1 (AP02461), -1TP2 (AP02462), -1TP3 (AP02463), -1TP4, (AP02464), -2TP1 (AP02465), -2TP2 (AP02466), -1LF1 (AP02468), -1LF2 (AP02469), -2LF1 (AP02470), -2LF2 (AP02476), Jingdongin-1-GN1 (AP02471), Palustrin-2GN1 (AP02472), -2GN2 (AP02473), -2GN3 (AP02474), Esculentin-2LF1 (AP02475)
Lithobates	119	*L. areolata* (formerly *Rana areolata*) (7): Temporin-1ARa (AP00864), Palustrin-2AR (AP01276), -3AR (AP01271), Esculentin-1ARa (AP01272), -1ARb (AP01273), Ranatuerin-2ARa (AP01274), -2ARb (AP01275)
		L. berlandieri (formerly *Rana berlandieri*) (8): Brevinin-1Ba (AP00463), -1Bb (AP00464), -1Bc (AP00465), -1Bd (AP00466), -1Be (AP00467), -1Bf (AP00468), Esculentin-2B (AP00662), Ranatuerin-2B (AP00120)
		L. blairi (3): Brevinin-1BLa (AP01412), -1BLb (AP01413), -1BLc (AP01414)
		L. capito (11): Temporin-CPa (AP01496), -CPb (AP01497), Brevinin-1CPa (AP01498), -1Wa (AP01499), Ranatuerin-2Wa (AP01500), -2Wb (AP01501), -2CPa (AP01505), -2CPb (AP01506), -2CPc (AP01507), Esculentin-2Wa (AP01502), -1CPa (AP01503)
		L. capito, L. sevosus (formerly *Rana sevosa*) (1): Esculentin-2SE (AP01504)
		L. catesbeianus (formerly *Rana catesbeiana*) (14): Temporin-La (AP02141), Palustrin-Ca (AP02142), Ranatuerin-1 (AP00114), -2 (AP00115), -3 (AP00116), -4 (AP00117), -5 (AP00659), -6 (AP00405), -7 (AP00406), - 8 (AP00407), -9 (AP00408), Ranalexin (AP00513), Cathelicidin-RC1 (AP02456), -RC2 (AP02457)
		L. chiricahuensis (6): Esculentin-2CHa (AP02111), Ranatuerin-2CHa (AP02112), -2CHb (AP02113), Brevinin-1CHa (AP02114), -1CHb (AP02115), -1CHc (AP02116)
		L. clamitans (formerly *Rana clamitans*) (10): Temporin-1Ca (AP00104), -1Cb (AP00105), -1Cc (AP00106), -1Cd (AP00107), -1Ce (AP00108), Ranatuerin-1C (AP00122), -2Cb (AP00123), Ranatuerin-2Ca (AP00124), Ranalexin-1Ca (AP00514), -1Cb (AP00515)
		L. grylio (formerly *Rana grylio*) (8): Temporin-1Ga (AP00823), -1Gb (AP00824), -1Gc (AP00825), -1Gd (AP00826), Ranatuerin-1Ga (AP00827), -1Gb (AP00828), -2G (AP00830), Ranalexin-1G (AP00829)
		L. heckscheri (formerly *Rana heckscheri*) (1): Temporin-1HKa (AP00868)
		L. okaloosae (formerly *Rana okaloosae*) (2): Temporin-1OLa (AP00871), -1OLb (AP00872)
		L. palustris (formerly *Rana palustris*) (22): Palustrin-1a (AP00614), -1b (AP00615), -1c (AP00616), -1d (AP00617), -2a (AP00618), -2b (AP00619), -2c (AP00620), -3a (AP00621), -3b (AP00622), Brevinin-1PLa (AP00646), -1PLb (AP00647), -1PLc (AP00648), Esculentin-1PLa (AP00649), -1PLb (AP00650), -2PLa (AP00651), Ranatuerin-2PLa (AP00652), -2PLb (AP00653), -2PLc (AP00654), -2PLd (AP00655), -2PLe (AP00656), -2PLf (AP00657), Temporin-1PLa (AP00658)
		L. pipiens (formerly *Rana pipiens*), *Rana pretiosa* (1): Ranatuerin-2P (AP00121)
		L. septentrionalis (formerly *Rana septentrionalis*) (9): Temporin-1SPa (AP01444), -1SPb (AP00598), Brevinin-1SPa (AP01438), -1SPb (AP01439), -1SPc (AP01441), -1SPd (AP01440), Ranatuerin-2SPa (AP01442), -2SPb (AP01443), Brevinin-2-related peptide (AP00599)
		L. sevosus (formerly *Rana sevosa*) (4): Ranatuerin-2SEb (AP00870), -2 SEc (AP00572), Esculentin-1SEa (AP00573), -1SEb (AP00604)

(Contd...)

Table 1. *(Continued)*

Anuran family[1] (Subfamily) Genus	Number of AMPs[2]	Species (number of AMPs): AMP (APD ID)[3]
		L. sphenacephalus (formerly *Rana sphenocephala*) (4): Brevinin-1SA (AP00453), -1SB (AP00454), -1SC (AP00455), Temporin-1S (AP02583)
		L. sylvaticus (formerly *Rana sylvatica*) (1): Brevinin-1SY (AP00452)
		L. virgatipes (formerly *Rana virgatipes*) (3): Temporin-1Va (AP00875), -1Vb (AP00876), -1Vc (AP01263)
		L. yavapaiensis (4): Brevinin-1Ya (AP01415), -1Yb (AP01416), -1Yc (AP01417), Ranatuerin-2Ya (AP01418)
Odorrana	158	*O. andersonii* (32): Brevinin-1-OA1 (AP01820), -1-OA2 (AP01821), -1-OA12 (AP01822), -2-OA1 (AP01834), -2-OA3 (AP01836), -2-OA4 (AP01837), -2-OA5 (AP01838), -2-OA6 (AP01839), -2-OA7 (AP01840), -2-OA8 (AP01841), Andersonin-C1 (AP01849), -D1 (AP01842), -W1 (AP01860), -W2 (AP01862), -X1 (AP01874), -Y1 (AP01824), Esculentin-1-OA1 (AP01855), -1-OA2 (AP01856), -1-OA3 (AP01857), -1-OA4 (AP01858), -1-OA5 (AP01859), -2-OA2 (AP01867), Nigrocin-1-OA1 (AP01875), -1-OA2 (AP01876), -1-OA3 (AP01877), Odorranain-A-OA1 (AP01886), -F-OA1 (AP01887), -F-OA2 (AP01888), -F-OA3 (AP01889), -F-OA4 (AP01890), -J-OA1 (AP01881), -J-OA2 (AP01869)
		O. andersonii, O. jingdongensis (1): Esculentin-2-OA1 (AP01866)
		O. grahami (formerly *Rana grahami*) (34): Ranacyclin-B3 (AP00490), -B5 (AP00491), Odorranain-HP (AP00600), -NR (AP00831), -B1 (AP01291), -C1 (AP01292), -D1 (AP01293), -E1 (AP01294), -F1 (AP01295), -G1(AP01296), -H1 (AP01297), -J1 (AP01298), -K1 (AP01299), -M1 (AP01300), -N1 (AP01301), -R1 (AP01302), -P2a (AP01304), -S1 (AP01305), -T1 (AP01306), -U1 (AP01307), -V1 (AP01308), -W1 (AP01309), Nigrocin-OG4 (AP01310), -OG5 (AP01311), -OG13 (AP01312), -OG20 (AP01313), -OG21 (AP01314), Palustrin-OG1 (AP02103), Esculentin-IGRa (AP00574), Brevinin-2GRb (AP00576), -1GRa (AP00578), Nigrocin-2GRa (AP00579), -2GRb (AP00580), -2GRc (AP00581)
		O. grahami, O. andersonii (3): Brevinin-2GRc (AP00577), Odorranain-P1a (AP01303), -2-OA2 (AP01835)
		O. grahami, O. jingdongensis (1): Brevinin-2GRa (AP00575)
		O. hainanensis (4): Temporin-HN1 (AP01959), -HN2 (AP01960), Brevinin-1HN1 (AP01961), -1V (AP01962)
		O. hejiangensis (2): Hejiangin-A1 (AP01891), -F1 (AP01892)
		O. hosii (7): Esculentin-1HSa (AP01419), -2HSa (AP01420), Brevinin-2HSa (AP01421), -2HSb (AP01422), -1HSa (AP01423), Nigrocin-2HSa (AP01425), -2HSa (AP01426)
		O. hosii, O. jingdongensis (1): Brevinin-1HSb (AP01424)
		O. ishikawae (13): Esculentin-1ISa (AP01703), -1ISb (AP01704), -2ISa (AP01705), Palustrin-2ISa (AP01706), -2SIb (AP01797), -2ISc (AP01781), Brevinin-2ISa (AP01707), -2ISb (AP01708), -2ISc (AP01709), -1ISa (AP01783), Nigrocin-2ISa (AP01710), -2ISb (AP01711), -2ISc (AP01782)
		O. jingdongensis (4): Brevinin-1JDa (AP02044), -1JDc (AP02045), Nigrocin-2JDa (AP02046), Odorranain-H2 (AP02047)
		O. livida (2): Nigrocin-2LVb (AP01535), Ranacyclin-B-RL1 (AP01908)
		O. livida, O. rotodora (4): Lividin-1 (AP01390), -2 (AP01391), -3 (AP01392), -4 (AP01393)
		O. rotodora (29): Brevinin-1-OR1 (AP01823), -1-OR3 (AP01825), -1-OR4 (AP01826), -1-OR5 (AP01827), -1-OR6 (AP01828), -1-OR7 (AP01829), -1-OR8 (AP01830), -1-OR9 (AP01831), -1-OR10 (AP01832), -1-OR11 (AP01833), -2-OR2 (AP01843), -2-OR3 (AP01844), -2-OR4 (AP01845), -2-OR5 (AP01846), -2-OR6 (AP01847), -2-OR7 (AP01848), -2-OR9 (AP01850), -2-OR10 (AP01851), Esculentin-1-OR1 (AP01861), -1-OR3 (AP01863), -1-OR4 (AP01864), -1-OR5 (AP01865), -2-OR2 (AP01870), -2-OR3 (AP01871), -2-OR4 (AP01872), -2-OR5 (AP01873), Nigrocin-1-OR1 (AP01878), -1-OR2 (AP01879), -1-OR3 (AP01880)

(Contd...)

Table 1. *(Continued)*

Anuran family[1] (Subfamily) Genus	Number of AMPs[2]	Species (number of AMPs): AMP (APD ID)[3]
		O. schmackeri (formerly *Rana schmackeri* (8): Brevinin-2HS1 (AP00880), -2HS2 (AP00881), -2HS3 (AP00882), -1HS2 (AP00883), Odorranain-P1a (AP01303), Nigrocin-2SCa (AP01536), -2SCc (AP01538), Schmackerin-C1 (AP01893)
		O. schmackeri, O. wuchuanensis (1): Nigrocin-2SCb (AP01537)
		O. versabilis (formerly *Rana versabilis*) (4): Esculentin-2VEb (AP00679), Ranatuerin-2VEb (AP00685), Nigrocin-2VB (AP01539), Temporin-1VE (AP00874)
		O. wuchuanensis (8): Brevinin-2-OW1 (AP01852), -2-OW2 (AP01853), -2-OW3 (AP01854), Odoranain-F-OW1 (AP01868), Nigrocin-1-OW2 (AP01882), -1-OW3 (AP01883), -1-OW4 (AP01884), -1-OW5 (AP01885)
Pelophylax	40	Hybrid *P. lessonae/ridibundus* (formerly *Rana esculenta*) (23): Brevinin-1Ea (AP00071), Brevinin-1Eb (AP00072), -1Ec (AP00862), -1Ed (AP01657), -1E (AP00073), -2EA (AP00076), -2EB (AP00077), -2EC (AP00078), -2ED (AP00079), Peptide A1 (AP00664), Peptide B9 (AP00665), CPRF-Ea (AP00814), -Eb (AP00815), -Ec (AP00816), Temporin-1Ec (AP00867), Esculentin-1 (AP00080), -1A (AP00081), -1B (AP00082), -1c (AP01656), -2A (AP00083), -2B (AP00084), Ranacyclin-T (AP01250), -E (AP01251)
		P. nigromaculatus (5): Nigrocin-1 (AP00508), -2 (AP00507), Temporin-1KM (AP02222), -1GY (AP02223), Pelophylaxin-2GY (AP02224)
		P. plancyi (formerly *P. plancyi fukienensis*) (4): Pelophylaxin-1 (AP01394), -2 (AP01395), -3 (AP01396), -4 (AP01397)
		P. porosus (formerly *Rana brevipoda porsa*) (2): Brevinin-1 (AP00074), -2 (AP00075)
		P. ridibundus (1): Brevinin-2R (AP02412)
		P. saharicus (5): Temporin-SHa (AP00898), -SHb (AP00899), -SHc (AP00900), -SHd (AP02118), -SHf (AP01534)
Rana	115	*R. aurora* (4): Temporin-1AUa (AP00865), Ranatuerin-2AUa (AP01435), Brevinin-1AUa (AP01436), -1AUb (AP01437)
		R. boylii (6): Temporin-1Bya (AP00866), Brevinin-1BYa (AP00878), Brevinin-1BYb (AP00885), -1BYc (AP00886), Brevinin-2BYa (AP00887), -2BYb (AP00888)
		R. cascadae (5): Ranatuerin-2CSa (AP00592), Brevinin-1CSa (AP00593), Temporin-1CSb (AP00595), -1CSc (AP00596), -1CSd (AP00597)
		R. chensinensis (10): Temporin-1CEa (AP01794), -1CEb (AP00605), -1CEc (AP01454), -1CEe (AP01921), Brevinin-1CDYa (AP01453), -2CE (AP01920), Japonicin-1CDYa (AP01455), Palustrin-2CE (AP01917), Chensinin-1CEb (AP01918), -3CE (AP01919)
		R. dalmatina (1): Brevinin-1Da (AP01771)
		R. draytonii (2): RV-23 (AP01264), Temporin-1DRa (AP01265)
		R. dybowskii (13): Dybowskin-1 (AP00564), -3 (AP00566), -4 (AP00567), -5 (AP00568), -6 (AP00569), Brevinin-1DYa (AP00601), -1DYb (AP00602), 1DYc (AP00603), -2DYa (AP00606), -2DYb (AP00607), -2DYd (AP00609), -2DYe (AP00610), Temporin-1DYa (AP00611)
		R. dybowskii, R. chensinensis (2): Dybowskin-2 (AP00565), Dybowskin-2CDYa (AP01550)
		R. japonica (4): Japonicin-1 (AP00357), -2 (AP00358), Temporin-1Ja (AP00869), Brevinin-1Ja (AP00877)
		R. luteiventris (5): Temporin-1La (AP00109), -1Lb (AP00110), -1Lc (AP00111), -1LB (AP00462), Esculentin-2L (AP00661)
		R. luteiventris, R. pretiosa (3): Ranatuerin-2La (AP00118), -2Lb (AP00119), Brevinin-1La (AP00461)

(Contd...)

Table 1. *(Continued)*

Anuran family[1] (Subfamily) Genus	Number of AMPs[2]	Species (number of AMPs): AMP (APD ID)[3]
		R. muscosa (2): Ranatuerin-2Ma (AP01410), -2Mb (AP01411)
		R. omeimontis (1): Japonicin-2OM1 (AP02416)
		R. ornativentris (6): Temporin-1Oa (AP00817), -1Ob (AP00818), -1Oc (AP00819), -1Od (AP00820), Brevinin-20a (AP00821), -20b (AP00822)
		R. pirica (8): Temporin-1PRa (AP00873), -1PRb (AP01259), Brevinin-2PRa (AP01253), -2PRb (AP01254), -2PRc (AP01255), -2PRd (AP01256), -2PRe (AP01257), Ranatuerin-2PRa (AP01258)
		R. pretiosa (9): Esculentin-2PRa (AP01716), -2PRa (AP01717), Ranatuerin-2PRb (AP01719), Brevinin-1PRa (AP01721), -1PRb (AP01722), -1PRc (AP01723), Temporin-PRa (AP01725), PRb (AP01726), -PRc (AP01727)
		R. sakuraii (5): Temporin-1SKa (AP01924), Ranatuerin-2SKa (AP01925), Peptide VR-23 (AP01926), Brevinin-2SKa (AP01928), -2SKb (AP01927)
		R. shuchinae (5): Shuchin-1 (AP01658), -2 (AP01659), -3 (AP02198), -4 (AP02199), -5 (AP02200)
		R. tagoi (3): AR-23 (AP00694), Temporin-1TGa (AP00695), -1TGb (AP01266)
		R. tagoi, R. sakuraii (1): Temporin-1TGc (AP01268)
		R. temporaria (16): Temporin-A (AP00094), -B (AP00095), -C (AP00096), -D (AP00858), -E (AP00097), -F (AP00098), -G (AP00099), -H (AP00859), -K (AP00100), -L (AP00101), Ranatuerin-1T (AP00113), Brevinin-1T (AP00456), -1Ta (AP00459), -1Tb (AP02166), -2TC (AP00457), -2TD (AP00458)
		R. tsushimensis (4): Brevinin-2TSa (AP00587), -1TSa (AP00588), Temporin-1TSa (AP00589), -1TSc (AP00591)
Sylvirana	21	*S. guentheri* (formerly *Hylarana guentheri*) (5): Brevinin-2GHa (AP00582), -2GHb (AP00583), -2GHc (AP00585), Guentherin (AP00584), Temporin-GH (AP00586)
		S. nigrovittata (formerly *Hylarana nigrovittata*) (5): Brevinin-2-RN1 (AP01644), -2-RN2 (AP01645), Ranacyclin-B-RN1 (AP01909), -B-RN2 (AP01910), -B-RN6 (AP01911)
		S. spinulosa (formerly *Hylarana spinulosa*) (11): Temporin-SN1 (AP02272), -SN2 (AP02273), -SN3 (AP02274), -SN4 (AP02275), Esculentin-2SN1 (AP02276), Brevinin-1SN1 (AP02278), -1SN2 (AP02279), -2SN1 (AP02280), -2SN2 (AP02281), -2SN3 (AP02282), -2SN4 (AP02277)
Rhacophoridae (Rhacophorinae) Rhacophorus	1	*R. puerensis* (formerly *Polypedates puerensis*) (1): PopuDef (AP02529)
Theloderma	2	*T. kwangsiense* (2): Pleurain-a1-thel (AP02260), Defensin-TK (AP02605)

[1]: Anuran families, genera and species are defined according to the classification of Amphibian Species of the World (http://research.amnh.org/vz/herpetology/amphibia/), [2]: The number of AMPs is based on the peptides listed in APD (the Antimicrobial Peptide Database, http://aps.unmc.edu/AP/main.php; last update: December 23, 2015). [3]: The name of AMPs and their APD ID link were taken from this database.

Ranidae) contains 23 distinct AMPs that are grouped into eight known peptide families (21). These are the brevinins-1, esculentins-1, esculentins-2, ranatuerins-2, temporins, palustrins-2, ranalexins, and the "orphan" peptides palustrins-1 (Table 3). The peptides contain a disulfide-bridged domain of varying sizes. The brevinin-1, esculentin-1, esculentin-2, and palustrin-2 families contain a 7-membered ring that has been referred to as the "Rana box". Peptides of the ranatuerin-2 family contain a 6-membered ring. Peptides of the temporin family do not contain a disulfide-bridged domain.

With few exceptions, each mixture of peptides includes several members of a particular AMP family that differ by only a few amino acid substitutions and/or deletions, and which are presumed to have arisen from multiple duplications of an ancestral gene (22, 23). Despite overlapping structural features, these isoforms often display marked differences in both the spectrum of activity and in potency against microorganisms (24). Moreover, orthologous as well as paralogous peptides exhibit synergy of action upon combination with other antibiotic molecules or AMPs, resulting in some cases

Table 2. Main AMP families and typical peptide sequences found in the skin of Anurans

Anuran family[1]	Typical genus[1]	AMP family	Typical peptide[1]	Amino acid sequence
Alsodidae	Alsodes	Ascaphin	Ascaphin-1M	GFRDVLKGAAKEFVKTVAGHIANa 2
Alytidae	Alytes	Alyteserin-1	Alyteserin-1c	GLKEIFKAGLGSLVKGIAAHVASa
		Alyteserin-2	Alyteserin-2a	ILGKLLSTAAGLLSNLa
Arthroleptidae	Leptopelis	Hylaseptin/Hylain	Hylaseptin-P1	GILDAIKAIAKAAG
Ascaphidae	Ascaphus	Ascaphin	Ascaphin-1	GFRDVLKGAAKAFVKTVAGHIANa
Bombinatoridae	Bombina	Bombinin	Bombinin	GIGALSAKGALKGLAKGLAEHFANa
		Bombinin H	Bombinin-H1	IIGPVLGMVG SALGGLLKKIa
Bufonidae	Bufo	Buforin	Buforin-I	AGRGKQGGKVRAKAKTRSSRAGLQFPVGRVHRLLRKGNY
Ceratophryidae	Ceratophrys	Ceratoxin	Ceratoxin	NVTPATKPTPSKPGYCRVMDELILCPDPPLSKDLCKNDS DCPGAQKCCYRTCIMQCLPPIFRE
Dicroglossidae	Hoplobatrachus	Tigerinin	Tigerinin-1	FCTMIPIPRCYa
Hylidae (Hylinae)	Hyla	Hylain/Hylaseptin	Hylain-1	GILDAIKAFANALG
	Hypsiboas	Raniseptin	Raniseptin-1	AWLDKLKSLGKVVGKVALGVAQNYLNPQQ
	Pseudis	Pseudin	Pseudin-1	GLNTLKKVFQGLHEAIKLINNHVQ
	Sphaenorhynchus	Frenatin	Frenatin-2.1.S	GLVGTLLGHIGKAILG
Hylidae (Pelodryadinae)	Litoria	Aurein	Aurein-2.1	GLLDIVKKVVGAFGSLa
		Caerin	Caerin-1.1	GLLSVLGSVAKHVLPHVVPVIAEHLa
		Citropin	Citropin-1.1	GLFDVIKKVASVIGGLa
		Dahlein	Dahlein-1.1	GLFDIIKNIVSTLa
		Maculatin	Maculatin-1.1	GLFGVLAKVAAHVVPAIAEHFa
Hylidae (Phyllomedusinae)	Phyllomedusa	Dermaseptin	Dermaseptin-S1	ALWKTMLKKLGTMALHAGKAALGAAADTISQGTQ
		Dermatoxin	Dermatoxin-B1	SLGSFLKGVGTTLASVGKVVSDQFGKLLQAGQa
		Phylloseptin	Phylloseptin-B1	FLSLIPHIVSGVAALAKHLa
		Phylloxin	Phylloxin-B1	GWMSKIASGIGTFLSGMQQa
		Plasticin	Plasticin-S1	GLVSDLLSTVTGLLGNLGGGGLKKI
		Orphan peptides	Dermaseptin-S9	GLRSKIWLWVLLMIWQESNKFKKM
Hyperoliidae	Kassina	Kassinatuerin	Kassinatuerin-1	GFMKYIGPLIPHAVKAISDLIa
Leptodactylidae (Leptodactylinae)	Leptodactylus	Ocellatin	Ocellatin-1	GVVDILKGAGKDLLAHLVGKISEKVa
Myobatrachidae	Crinia	Signiferin	Signiferin-2.1	IIGHLIKTALGMLGLa
	Uperoleia	Uperin	Uperin-3.1	GVLDAFRKIATVVKNVVa
Pipidae	Xenopus	Procaerulein-derived peptide	CPF-1	GFGSFLGKALKAALKIGANALGGSPQQ
		Magainin	Magainin-2	GIGKFLHSAKKFGKAFVGEIMNS
		Peptide glycine-leucine amide	PGLa	GMASKAGAIAGKIAKVALKALa
		Proxenopsin-derived peptide	XPF-1	GWASKIGQTLGKIAKVGLKQLIQPK

(Contd...)

Table 2. *(Continued)*

Anuran family[1]	Typical genus[1]	AMP family	Typical peptide[1]	Amino acid sequence
Ranidae	Rana	Brevinin-1	Brevinin-1	FLPVLAGIAAKVVPALFCKITKKC[3]
		Brevinin-2	Brevinin-2	GLLDSLKGFAATAGKGVLQSLLSTASCKLAKTC
		Esculentin-1	Esculentin-1SEb	GLFSKFNKKKIKSGLFKIIKTAGKEAGLEALRT GIDVIGCKIKGEC
		Esculentin-2	Esculentin-2PLa	GLFSILKGVGKIALKGLAKNMGKMGLDLVSCKISKEC
		Japonicin-1	Japonicin-1J	FFPIGVFCKIFKTC
		Japonicin-2	Japonicin-2J	FGLPMLSILPKALCILLKRKC
		Nigrocin-2	Nigrocin-2GRb	GLFGKILGVGKKVLCGLSGMC
		Palustrin-2	Palustrin-2PLa	GFLSTVKNLATNVAGTVLDTIRCKVTGGCRP
		Ranacyclin	Ranacyclin-T	GALRGCWTKSYPPKPCKa
		Ranatuerin-1	Ranatuerin-1CBa	SMLSVLKNLGKVGLGFVACKINKQC
		Ranatuerin-2	Ranatuerin-2CHa	GLMDTVKNAAKNLAGQLLDRLKCKITGC
		Temporin	Temporin-SKa	FLPVILPVIGKLLNGILa

[1]: Names of frog species and peptides follow the frog species names set out in Amphibian Species of the World (http://research.amnh.org/vz/herpetology/amphibia/) and recent proposed guidelines for antimicrobial peptide nomenclature (15-18), [2]: a=C-terminal amidation, [3]: Cysteine residues in bold letters are disulfide bridged

Table 3. Intraspecific diversity of antimicrobial peptides from frog skin secretions

Frog species[1]	Peptide family	Peptide[1]	Amino acid sequence
Phyllomedusa sauvagii (Familly: Hylidae)	Dermaseptin	Dermaseptin-S1	ALWKTMLKKLGTMALHAGKAALGAAADTISQGTQ
		Dermaseptin-S2	ALWFTMLKKLGTMALHAGKAALGAAANTISQGTQ
		Dermaseptin-S3	ALWKNMLKGIGKLAGKAALGAVKKLVGAES
		Dermaseptin-S4	ALWMTLLKKVLKAAAKALNAVLVGANA
		Dermaseptin-S5	GLWSKIKTAGKSVAKAAAKAAVKAVTNAV
		Dermaseptin-S6	GLWSKIKTAGKEAAKAAAKAAGKAALNAVSEAIa[2]
		Dermaseptin-S7	GLWKSLLKNVGKAAGKAALNAVTDMVNQa
		Dermaseptin-S8	ALWKTMLKKLGTVALHAGKAALGAAADTISQa
		Dermaseptin-S11	ALWKTLLKGAGKVFGHVAKQFLGSQGQPES
		Dermaseptin-S12	GLWSKIKEAAKTAGKMAMGFVNDMVa
		Dermaseptin-S13	GLRSKIKEAAKTAGKMALGFVNDMAa
	Dermatoxin	Dermatoxin-S1	ALGTLLKGVGSAVATVGKMVADQFGKLLQAa
	Phylloseptin	Phylloseptin-S1	FLSLIPHIVSGVASIAKHFa
		Phylloseptin-S2	FLSLIPHIVSGVASLAKHFa
		Phylloseptin-S3	FLSLIPHIVSGVASLAIHFa
		Phylloseptin-S4	FLSMIPHIVSGVAALAKHLa
		Phylloseptin-S5	LLGMIPVAISAISALSKLa
		Phylloseptin-S6	FLSLIPHIVSGVASIAKHLa
	Phylloxin	Phylloxin-S1	GWMSKIASGIGTFLSGVQQa
	Plasticin	Plasticin-S1	GLVSDLLSTVTGLLG NLGGGGLKKI
	Orphan peptide	Dermaseptin-S9	GLRSKIWLWVLLMIWQESNKFKKM

(Contd...)

Table 3. *(Continued)*

Frog species[1]	Peptide family	Peptide[1]	Amino acid sequence
Lithobates (*Rana*) *palustris* (Familly: Ranidae)	Brevinin-1	Brevinin-1PLa	FFPNVASVPGQVLKKIFCAISKKC[3]
		Brevinin-1PLb	FLPLIAGLAANFLPKIFCAITKKC
		Brevinin-1PLc	FLPVIAGVAAKFLPKIFCAITKKC
	Esculentin-1	Esculentin-1PLa	GLFPKINKKKAKTGVFNIIKTVGKEAGMDLIRTGIDTIGCKIKGEC
		Esculentin-1PLb	GIFTKINKKKAKTGVFNIIKTIGKEAGMDVIRAGIDTISCKIKGEC
	Esculentin-2	Esculentin-2PLa	GLFSILKGVGKIALKGLAKNMGKMGLDLVSCKISKEC
	Orphan peptides	Palustrin-1PLa	ALFSILRGLKKLGKMGQAFVNCEIYKKC
		Palustrin-1PLb	ALFSILRGLKKLGNMGQAFV NCKIYKKC
		Palustrin-1PLc	ALSILRGLEKLAKMGIALTNCKATKKC
		Palustrin-1PLd	ALSILKGLEKLAKMGIALTN CKATKKC
	Palustrin-2	Palustrin-2PLa	GFLSTVKNLATNVAGTVLDTIRCKVTGGCRP
		Palustrin-2PLb	GFFSTVKNLATNVAGTVIDTLKCKVTGGCRS
		Palustrin-2PLc	GFLSTVKNLATNVAGTVIDTLKCKVTGGCRS
	Ranalexin	Ranalexin-PLa	SVIGCWKTKSIPPRPCFFK
		Ranalexin-PLb	LIRGCWTKSIPPKCPLV
		Ranalexin-PLc	SVIGCWKTKSIPPRPCFV
	Ranatuerin-2	Ranatuerin-2PLa	GIMDTVKNVAKNLAGQLLDKLKCKITAC
		Ranatuerin-2PLb	GIMDTVKNAAKDLAGQLLDKLKCRITGC
		Ranatuerin-2PLc	GLLDTIKNTAKNLAVGLLDKIKCKMTGC
		Ranatuerin-2PLd	GIMDSVKNVAKNIAGQLLDKLKCKITGC
		Ranatuerin-2PLe	GIMDSVKNAAKNLAGQLLDTIKCKITAC
		Ranatuerin-2PLf	GIMDTVKNAAKDLAGQLDKLKCRITGC
	Temporin	Temporin-1PLa	FLPLVGKILSGLIa

[1]: Names of frog species and peptides follow the frog species names set out in Amphibian Species of the World (http://research.amnh.org/vz/herpetology/amphibia/) and recent proposed guidelines for antimicrobial peptide nomenclature (15-18), [2]: a=C-terminal amidation, [3]: Cysteine residues in bold letters are disulfide bridged

in a 100-fold increase in antibiotic activity potency over the activity of the peptides separately (24, 25). Such a complex mixture of antibiotic peptides, with different specificity and potency within a frog species, behaves as a multi-drug defense system providing frogs with the maximum protection at a minimum metabolic cost (26) against a broad array of microorganisms and minimizing the chance of microorganisms developing resistance to individual peptides.

3.2. Interspecies diversity of frog skin AMPs: different species of frogs are equipped with different sets of peptides belonging to different families

Amphibians belonging to different families, genera, species or even subspecies store distinct sets of peptides belonging to different AMP families. Orthologous peptides, as well as paralogous peptides, have different amino acid sequences in different species, and often display different biological activity (27, 28). The impressive divergence between frog species is such that no two species, even those species that are closely related phylogenetically, have yet been found that have the same panoply of peptide antibiotics, and no single peptide from one species has been found with an identical amino acid sequence in another.

For instance, 74 distinct peptides from the dermaseptin family have been described from the skins of fifteen Phyllomedusinae species (Table 4) (19). Although they vary in length, amino acid sequence, hydrophobicity, and charge distribution, they all share a signature pattern consisting of a conserved Trp residue at position 3 and an AA(A/G)KAAL(G/N)A consensus motif in the midregion. These peptides are prototypical members of a large class of membrane-damaging cationic peptides

Table 4. Examples of interspecific diversity of dermaseptins from hylid frogs and temporins from ranid frogs

Anuran family	Genus species[1]	Peptide[1,2]	Amino acid sequence
Hylidae		Dermaseptin	
	Agalychnis		
	A. annae	Dermaseptin-A3	SLWSKIKEMAATAGKAALNAVTGMVNQa[3]
	A. callidryas	Dermaseptin-C1	GMWSKIKEAGKAAAKAAAKAAGKAALDVVSGAIa
	A. dacnicolor	Dermaseptin-DA2	ALWKTLLKKVGKVAGKAVLNAVTNMANQNEQ
	A. lemur	Dermaseptin-L1	GLWSKIKEAAKAAGKAALNAVTGLVNQGDQPS
	A. spurrelli	Dermaseptin-LI1	AVWKDFLKNIGKAAGKAVLNSVTDMVNE
	Phasmahyla		
	P. jandaia	Dermaseptin-J1	GLWKNMLSGIGKLAGQAALGAVKTLVa
	Phyllomedusa		
	P. bicolor	Dermaseptin-B1	AMWKDVLKKIGTVALHAGKAALGAVADTISQa
	P. burmeisteri	Dermaseptin-BU1	ALWKNMLKGIGKLAGKAALGAVK
	P. distincta	Dermaseptin-DI1	GLWSKIKAAGKEAAKAAAKAAGKAALNAVSEAV
	P. hypochondrialis/azurea	Dermaseptin-H3	GLWSTIKNVAAAAGKAALGALa
	P. oreades	Dermaseptin-O1	GLWSTIKQKGKEAAIAAAKAAGQAALGALa
	P. sauvagii	Dermaseptin-S1	ALWKTMLKKLGTMALHAGKAALGAAADTISQGTQ
	P. tarsius	Dermaseptin-TA1	GLWSKIKETGKEAAKAAGKAALNKIAEAVa
	P. tomopterna	Dermaseptin-TO1	ALWKDLLKNVGIAAGKAVLNKVTDMVNQa
	P. trinitatis	Dermaseptin-TR1	ALWKDILKNVGKAAGKAVLNTVTDMVNQa
Ranidae		Temporin	
	Amolops		
	A. chunganensis	Temporins-CG1	FLPFVGNLLKGLLa
	A. jinjiangensis	Temporin-AJ1	FLPIVTGLLSSLLa
	A. loloensis	Temporin-ALd	FLPIAGKLLSGLSGLLa
	"Hylarana"		
	"H." latouchii	Temporin-LT1	FLPGLIAGIAKMLa
	Lithobathes		
	L. capito	Temporin-CPb	FLPIVGRLISGILa
	L. palustris	Temporin-1PLa	FLPLVGKILSGLIa
	L. pipiens	Temprorin-1P	FLPIVGKLLSGLLa
	Odorrana		
	O. andersonii	Temporin-1-RA1	FLFPLAKASFLGKVLa
	O. hainanensis	Temporin-HN1	AILTTLANWARKFLa
	O. versabilis	Temporin-1Ve	FLPLVGKILSGLIa
	Pelophylax		
	P. hubeiensis	Temporin-HB1	FLPLLAGLAAKWFa

(Contd...)

Table 4. *(Continued)*

Anuran family	Genus species[1]	Peptide[1,2]	Amino acid sequence
	P. nigromaculatus	Temporin-1RNa	ILPIRSLIKKLLa
	P. saharicus	Temporin-SHa	FLSGIVGMLGKLFa
	Rana		
	R. amurensis	Temporin-1AM	FLPLVGKILSGLIa
	R. boylii	Temporin-1BYa	FLPIIAKVLSGLLa
	R. sierrae	Temporin-1SR	FLPIIAKVLGNLLa
	Sylvirana		
	S. guentheri	Temporin-GH	FLPLLFGAISHLLa
	S. spinulosa	Temporin-SN1	FFPFLLGALGSLLPKIFa

[1]: Names of frog species and peptides follow the frog species names set out in Amphibian Species of the World (http://research.amnh.org/vz/herpetology/amphibia/) and recent proposed guidelines for antimicrobial peptide nomenclature (15-18), [2]: Only a single representative member is shown for each frog species, [3]: a=C-terminal amidation.

that undergo coil-to-helix transition on binding to lipid bilayers. Most are cidal at micromolar doses against a wide spectrum of microorganisms, including wall-less bacteria, Gram-negative and Gram-positive bacteria, fungi, yeast, but each peptide has considerable variations in its antimicrobial activity. All the dermaseptins, except DRS-S4, are not or weakly toxic against mammalian cells. Dermaseptins S1–S5 *from Phyllomedusa sauvagii*, DRS-O1 from *P. oreades* (or DRS-01) and DRS-H4 (or DShypo 01) from *P. hypochondrialis/azurea* exhibit cidal activity against *Leishmania* (promastigote form) at micromolar doses (29-32). DRS-S3 and derivatives of DRS-S4 selectively disrupt the plasma membrane of the intracellular parasite *Plasmodium falciparum* without harming that of the mammalian host cell (33). Dermaseptin-O1, and dermaseptin-DI1 and -DI2 (*Phyllomedusa distincta*) show antiprotozoan activity against *Trypanosoma cruzi* in its trypomastigote and epimastigote forms cultivated in both cell culture and blood media, without toxicity against mouse erythrocytes and white blood cells (34). Dermaseptin 1 and 4 (*Phyllomedusa nordestina*) display also anti-Trypanosoma activity against *T. cruzi* trypomastigotes without toxicity toward peritoneal macrophages (32). Dermaseptins S1–S5 display antiviral activity against herpes simplex virus type I and HIV-1 virus at micromolar doses by disrupting the virion integrity (35). Dermaseptin-S4 analogs have a potent activity against human sperm (36).

Temporins constitute a family of highly hydrophobic and weakly charged linear antimicrobial peptides (AMPs) that are widely distributed in North American and Eurasian frogs of the Ranidae family (10, 37). With more than 100 members, temporins represent one of the largest AMP families among the 12 different families that have been identified in the skins of ranid frogs (Table 2). Temporins are among the smallest cytotoxic peptides found in nature, with 8-17 residues, and the most highly variable of all AMPs (Table 4). Like dermaseptins, temporins form amphipathic alpha-helices with alternating hydrophobic and polar residues in apolar media or membrane mimetic environments (10, 38-42). In most cases, there is a direct correlation between the antimicrobial potency and the net positive charge of temporins. Most naturally occurring temporins contain a single basic amino acid residue and show potent antimicrobial activity against Gram-positive bacteria, but are inactive, or weakly active against Gram-negative bacteria. Temporin L (FVQWFSKFLGRIL$_{amide}$) from *Rana temporaria*, temporin-DRa (HFLGTLVNLAKKIL$_{amide}$) from *Rana draytonii*, temporin-CPa (IPPFIKKVLTTVF$_{amide}$) from *Lithobates capito*, which bear a net charge of +3, and temporin-SHa (FLSGIVGMLGKLF$_{amide}$) from *Pelophylax saharicus*, which bear a net charge of +2, exhibit broad-spectrum activity against Gram-positive and Gram-negative bacteria, and yeasts (40, 43-48). However, most of these peptides are hemolytic against human erythrocytes. Temporin-SHf (FFFLSRIF$_{amide}$, net charge = +2) from *Pelophylax saharicus* (42) is a unique case among frog skin AMPs. It is the smallest linear antimicrobial peptide found to date in vertebrates, with only 8 residues. It has a highly hydrophobic sequence and possesses the highest percentage of Phe residues of any known peptide or protein. Despite its small size, temporin-SHf kills Gram-positive bacteria and yeasts by permeating/disrupting the microbial membrane, with no hemolytic activity. A few temporins, temporin-Ta (FLPLIGRVLSGIL$_{amide}$), -Tb (LLPIVGNLLKSLL$_{amide}$), -Tf (FLPLIGKVLSGIL$_{amide}$) and -Tl (FVQWFSKFLGRIL$_{amide}$) from *Rana temporaria*, and temporin-SHa and -SHd (FLPAALAGIGGILGKLF$_{amide}$) from *Pelophylax saharicus* have also leishmanicidal activity at concentrations that are not or weakly toxic to macrophages (43, 49, 50, 51).

These few representative examples clearly show that the phenomenal interspecies and intraspecies divergences of antimicrobial peptide sequences are dominant factors in the differences observed between the frog peptide arsenals. The peptide-rich secretion of the skin of frogs and toads is thus a natural chemical library that may be readily exploited to discover new antimicrobial agents targeting specific microorganisms for which the therapeutic armamentarium is scarce. Also, the discovery of new members of known peptide families is not of minor importance since they may shed light on the exact roles of various parameters, such as net charge, percent of helical/beta-structure, amphipathy and conformational flexibility, on the ability of AMPs to bind to and disrupt bacterial membranes, and how these peptides may be used to design or optimize new active agents.

3.3. Molecular strategies to generate antimicrobial peptide diversity in frog skin

What is the strategy that these frogs have evolved to generate this enormous array of peptides? We currently have a limited amount of data mainly from antimicrobial peptides that are made in the skin of Hylidae, Ranidae, Hyperoliidae and Dicroglossidae frogs. Peptides are initially synthesized in the multi-nucleated cells of the granular glands of the skin through ribosomal translation as tripartite prepropeptides, post-translationally processed to yield the mature biologically active peptide, and then stored in the large granules of the glands (52, 53). As a general rule, each polypeptide precursor results from transcription of a single genetic locus that encodes a single mature peptide. Precursors typically have an N-terminal signal sequence of 22 amino acids, an acidic intervening sequence that ends in a typical prohormone processing signal Lys-Arg, and at the C-terminal end, the mature antimicrobial peptide in single copy (Figure 2). This rather conventional prepropeptide organization masks a striking and unconventional evolutionary pattern. Comparisons between the precursor sequences reveal an extraordinary juxtaposition; signal sequences and acidic intervening sequences are conserved to an unprecedented degree both within and between frog species, even in the case of frogs belonging to distant families, but the C-terminal regions corresponding to antimicrobial peptides vary markedly (23). For instance, antimicrobial peptides from Eurasian and North American ranid frogs that belong to the brevinins, esculentins, nigrocins, palustrins, ranacyclin, ranatuerins, japonicins and temporin families have precursors whose preproregions strikingly resemble those of tigerinins and limnonectins from Dicroglossidae frogs, kasseptins and galensins from African Hyperoliidae frogs, caerins, fallaxidins, frenatins, aureins from Australian Pelodryadinae frogs (family Hylidae), and hylaseptins and raniseptins from Hylinae frogs (family Hylidae), as well as those of dermaseptins, dermatoxins, phylloseptins, phylloxins and plasticins from South American Hylidae frogs (19). The contrast between the strikingly conserved preproregion and the hyperdivergent mature peptides is one of the most extreme examples observed to date for homologous gene products within a single order of organisms. There is little information available regarding the gene organization of most AMPs. Genes encoding dermaseptin B2 and phylloxin from *Phyllomedusa bicolor* are highly conserved in terms of structural organization with a small intron of 137- and 175-bp, respectively, located between exon 1 (encoding the signal peptide and the first three residues of the acidic intervening sequence) and exon 2 (remainder of acidic intervening sequence and mature peptide coding sequence through into 3′-non-translated region) (54, 55). The structural organization and intron/exon boundary are preserved in the gene that encodes gaegurin-4 (new nomenclature: brevinin-2EMd) from *Rana rugosa* (*Glandirana emeljanovi*) but with an intronic sequence of 3.5 kb (56).

The remarkable similarity of preproregions of precursors that give rise to very different end products in distantly related frogs suggests that all the corresponding genes came from a common ancestor in the ancestor of South American and Australian hylids and North American and Eurasian ranids before their divergence in the Mezozoic age 150 million years ago (57). A combination of phylogenetic reconstructions, analysis of mutation rates, and geophysical models for the sequence of fragmentation of Gondwana suggest that the impressive diversity of these peptides and the number of peptides per species reflect the combination of multiple duplications of the ancestral gene before and during radiation of these species and within individual species, focal hypermutations of the mature peptide domain, and subsequent actions of diversifying (positive) selection (9, 23, 58-60). South American and Australian hylids, as well as Indian, European, Asian and North American ranids, have different patterns of distribution with respect to geography, climate, vegetation and habitats (aquatic, semi-aquatic, terrestrial, arboreal, torrential, fosserial, rocky), some of them showing very unusual and extreme adaptations. Massive gene duplication and targeted hypermutation events of the C-terminal antimicrobial-coding region might have evolved as a way of increasing genetic diversity, and so accelerating the adaptation of frogs to noxious microbial fauna when microbial predators changed very rapidly with shifts to new ecological niches. Frog skin secretions are a rich source of biologically active neuropeptides and hormones, which co-occur with AMPs. Most interesting, several neuropeptide and hormone families have precursors with a signal sequence and acidic propiece that show a high degree of similarity to those found in AMP precursors (e.g., opioid peptides belonging to the dermorphin/deltorphin family, tachykinins, bradykinins, hyposins, tryptophyllins, and Bowman-Birk-like trypsin inhibitors) (9). The discovery of considerable extent

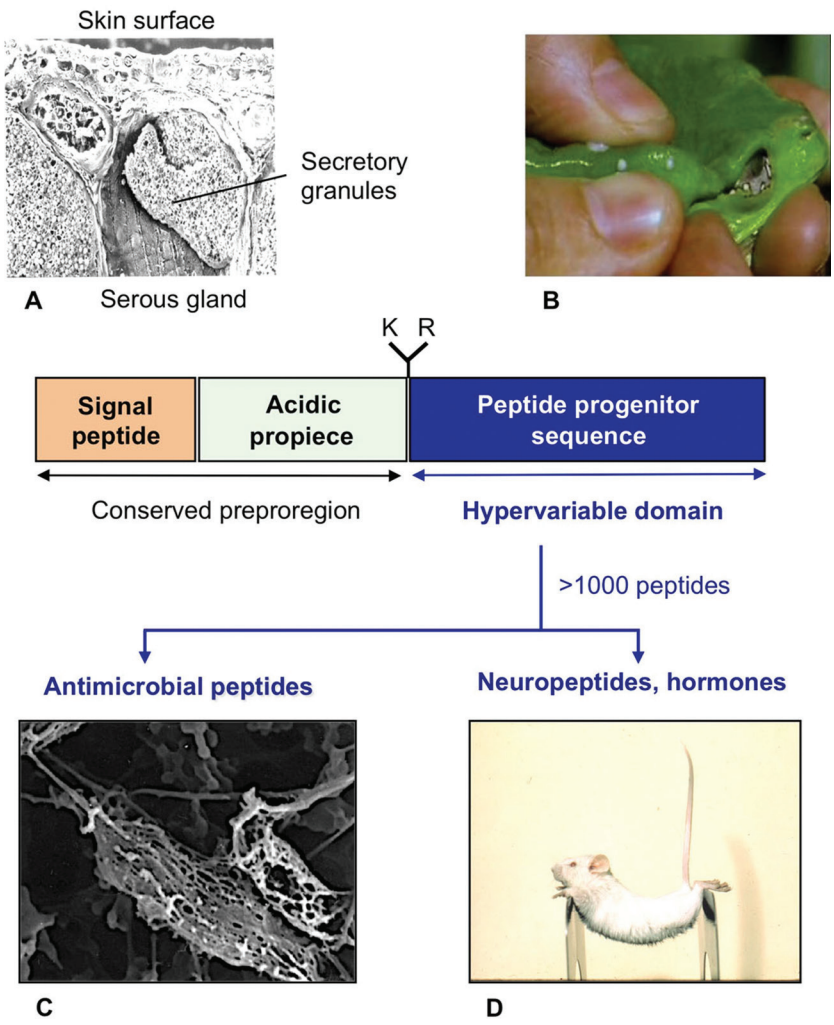


Figure 2. Schematic representation of the canonical precursor architecture of antimicrobial peptides and neuropeptides from Hylidae and Ranidae frogs. The coding region, including the signal peptide, the acidic propiece and the peptide progenitor sequence is drawn as a rectangle. The signal peptide includes the first 22 amino acid residues, while the acidic propiece comprises 19–26 residues. Lys-Arg is the site of cleavage of prohormone connvertases. A) Peptides are initially synthesized in the multi-nucleated cells of the serous glands of the skin through ribosomal translation as tripartite prepropeptides, post-translationally processed to yield the mature biologically active peptide, and then stored in the large granules of the glands. Glands may release their content onto the skin surface by a holocrine mechanism involving the rupture of the plasma membrane and the extrusion of the secretory granules through a duct opening to the surface. B) Peptide exudate can be recovered by gentle squeezing of the latero-dorsal portion of the skin. C) Electron microscopic observations of *Leishmania mexicana* promastigotes after treatment with 5 μM dermaseptin-S1 (Drs-S1). Within 5 min of incubation in the presence of Drs-S1, the flagellated parasites lost their motility. After treatment, the plasma membrane is peeled off, and the microtubular network is the unique cell surface structure that still maintains the shape and integrity of the promastigote ghost. D) Intracerebroventricular administration of dermorphin (YaFGYPS$_{amide}$; a = d-Ala residue), a d-amino acid containing-opioid peptide, elicits analgesia and catalepsy in mice.

of sequence identities between the neuropeptide and antimicrobial peptide precursors is unprecedented. Thus, although the two groups of peptides are genetically related and belong to the same gene superfamily, they have strongly diverged to yield families of peptides that are both structurally and functionally distinct.

Today, there are more than 6500 described anuran species in the world (Amphibian Species of the World, http://research.amnh.org/vz/herpetology/amphibia/). According to Conlon, the distribution of AMPs among these species is sporadic, being restricted to certain families and to certain genera (8). The lack of AMPs in a number of frog species, even those living in environments laden with microbes, may cast doubt on the exact physiological role that these peptides play in frog skin (8). As stated by Shaw (3), the cytolytic action of some AMPs on vertebrate cells and their coexpression with neuropeptides in several frog families may indicate that they would assist neuropeptides in their anti-predator role by increasing the delivery of the latter to the endocrine and nervous system of the predators. Information about anuran AMPs in archaeobatrachian frogs is much more sparse. To date, the limited DNA sequence data

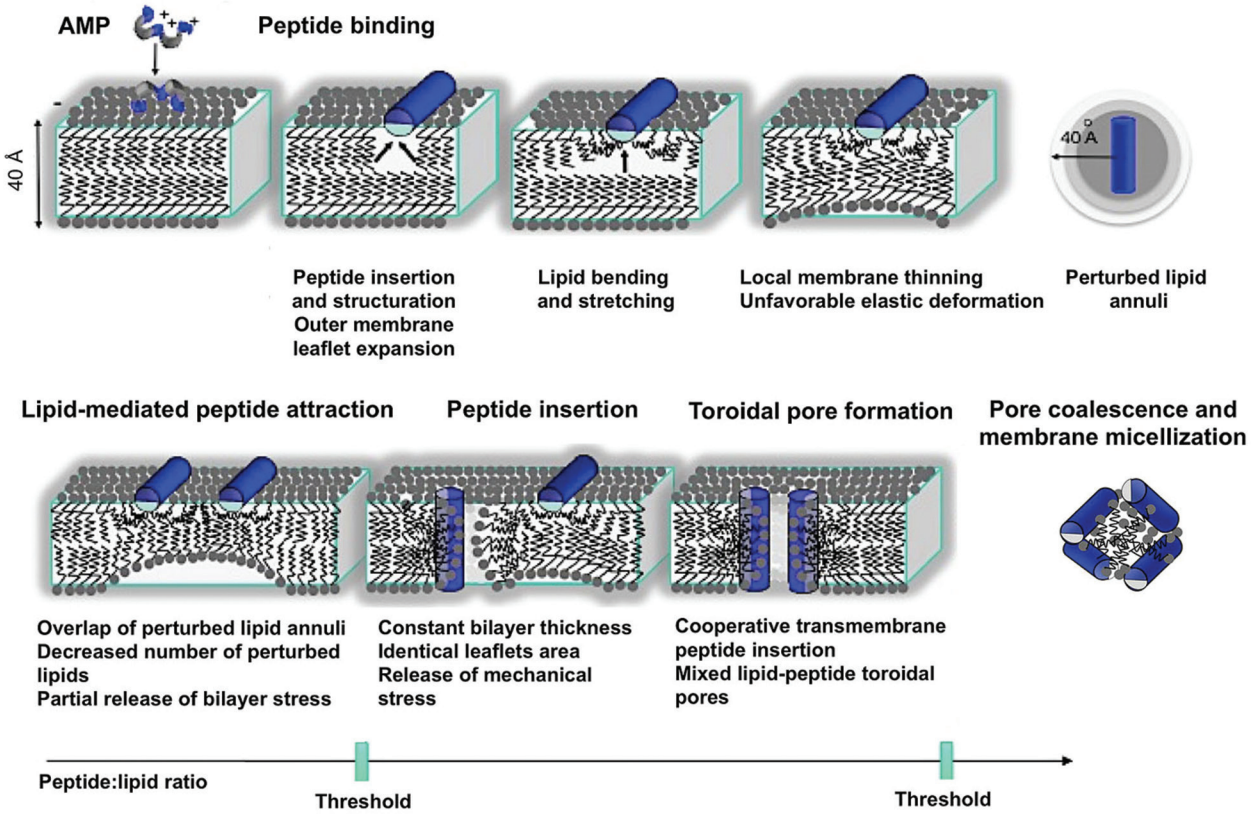


Figure 3. A proposed mechanism for bacterial membrane permeation/disruption by alpha-helical antimicrobial peptides from frog skin.The cationic peptide reaches the negatively charged outer leaflet of the bacterial membrane by long-range electrostatic interactions and binds to the surface of the membrane with its hydrophobic surface facing the membrane and its hydrophilic surface facing the solvent. The subsequent formation of an amphipathic peptide helix causes membrane thinning and positive curvature strain. When a threshold concentration of peptide is reached, a fraction of the peptides change their orientation from parallel to transversal to release the strain, hence forming transient mixed phospholipid-peptide toroidal pores that permeate the membrane. When a second threshold concentration of peptide is reached, toroidal pores fuse together, leading to membrane disruption or disintegration in a detergent-like manner.

regarding biosynthesis of AMPs in Archaeobatrachia show that the signal sequences of the polypeptide precursors corresponding to the evolutionary lineages Bombinatoridae (*Bombina* spp.), Pipidae (*Xenopus laevis*) and Alytidae (*Alytes* spp.) are strongly conserved within each lineage, but highly divergent between them and between the Hylidae lineage (59).

3.4. Mechanisms of microbicidal activity

The mode of antimicrobial action of most of the frog skin AMPs is believed to be the permeation/disruption of the lipid plasma membrane of the target cells through binding of cationic peptides to the outer leaflet of bacterial bilayers (Figure 3). The high content of anionic lipids in prokaryotic membranes and their absence from the neutral matrix of erythrocytes account for the preferential binding of cationic peptides to bacterial membranes through long-range electrostatic interactions. The Shai-Matzusaki-Huang unifying model (11-14) proposes: (i) that the cationic peptide binds, parallel to the membrane plane, to the phospholipid head groups and cover the membrane with the apolar amino acids penetrating partly into the bilayer hydrocarbon core and the cationic residues interacting with the negatively charged phosphate moieties of the lipid headgroups, (ii) subsequent formation of an amphipathic peptide helix; (iii) rotation of the helical peptides leading to reorientation of the hydrophobic residues toward the hydrophobic core of the membrane. The resulting spatial segregation of polar and apolar amino acids on opposing faces along the long axis of the helix permits the insertion of a well-defined hydrophobic sector into the acyl core of the lipid bilayer, hence causing membrane thinning and positive curvature strain; (iv) to release the strain, a fraction of the peptides changes their orientation from parallel to transversal, forming transient mixed phospholipid-peptide toroidal pores. Note that only a few pores exist after the redistribution of the peptides between the two leaflets, because the pore formation is a cooperative process. Therefore, the integrity of the membrane is only transiently breached, and (v) once a threshold accumulation of membrane-bound peptide is reached, this may lead to disruption/solubilization of the membrane in a detergent-like manner. The threshold between the toroidal pore

and the detergent-like mechanisms of action may be two facets of the cell killing mechanism relying on the peptide concentration, the membrane composition, and the final peptide-to-lipid ratio.

There is a widespread acceptance that cationic antimicrobial peptides, aside their membrane permeabilizing/disrupting properties, may also operate through interaction with intracellular targets, or disruption of key cellular processes (inhibition of DNA and protein synthesis, inhibition of chaperone-assisted protein folding and enzymatic activity, and inhibition of cytoplasmic membrane septum formation and cell wall synthesis). From the limited data currently available, it seems unlikely that this property is shared by the hundreds of naturally occurring AMPs that differ by length, amino acid composition, sequence, hydrophobicity, amphipathicity, and membrane-bound conformation (14).

3.5. Concluding remarks

Scorpions, spiders, cone snails, amphibians, and snakes produce toxic peptides for defense, competitor deterrence, or prey capture. The repertoire of toxic peptides encoded in the genomes of these animals is phenomenal (61). It has been estimated that the 500 species of cone snails express 50,000 different neurotoxins (62, 63). Scorpions have been estimated to encode a similar diversity of peptide neurotoxins (100,000) based on a species count of 1500 (64). Spiders, with 80,000 estimated species lead to a total of 4 million spider-venom polypeptides. As stated by Olivera (65), gene products that mediate interactions between organisms are encoded by exogenes, a term used to rationalize conotoxin gene hypermutation. Exogene families that are targeted to other organism in the environment, such as the conopeptide genes, have to diversify rapidly because each species has its own ecological niche. In consequence, the evolutionary history of these genes would be expected to be strikingly different from the rest of the genome. This suggest that spiders, scorpions, cone snails and frogs, have independently evolved a similar mechanism to diversify their repertoire of toxic polypeptides through duplication of ancestral gene(s), followed by action of a hypervariability-generating mechanism that increases the rate of mutation of the mature-toxin loci. The pool of resulting paralogous genes was then subject to adaptive evolution to select useful variants, enabling these animals to perform a comprehensive search of pharmacophore space (61).

4. THERAPEUTIC PROMISES

The use of AMPs as a therapeutic alternative to conventional antibiotics has attracted considerable scientific interest in the 1990s. Indeed, broad-spectrum activity, rapid permeabilization/disruption of the target cell membrane, and low possibility for microbes to develop resistance endowed peptide-based antimicrobials with attractive advantages over classical antibiotics. However, the strong initial commitment of biotech companies and pharmaceutical industries into AMPs development suffered a sudden stop in 1999 when the US Food and Drug Administration (FDA) denied marketing approval of pexiganan (66), also known as MSI-78 (a 22-residue linear peptide analogue of magainin-2 (67) identified from the skin of *Xenopus laevis*), after completion of two phase III clinical trials for topical treatment of infected foot ulcers in diabetic patients, arguing that the peptide did not show superior efficacy over the conventional treatment with ofloxacin, a fluoroquinolone antibiotic. In addition, developers and pharma partners faced significant challenges associated with AMPs. As peptides, AMPs have many limitations for drug development including a very high manufacturing costs as compare to that of small molecules, a low oral bioavailability, short half-life owing to rapid renal clearance, lack of stability due to protease degradation, potential immunogenicity, toxicity to healthy host cells, and difficulties into formulation and manufacturing (68, 69). Another setback that has hindered the development of AMPs was their poor efficacy against Gram-negative bacteria and bacteria that form biofilms for which the therapeutic armamentarium is scarce.

In recent years, however, biotech companies and the pharmaceutical industry are investing more and more in the development of peptide-like antimicrobial drugs for use in humans (70). This renewed interest in commercial development of AMPs resulted mainly from i/technological breakthrough and advances accomplished in the last decade in the development of innovative strategies for bulk production at low cost (kg to multi-tonne per year, US $7.5-10 to <US $1 per gram per amino acid residue, respectively) of peptides of 5-50 amino acids by chemical synthesis, peptide drug delivery techniques (oral, nasal, sublingual, pulmonary, transdermally by high-pressure propellant gas or patch), and peptide protection (cyclization, encapsulation in biodegradable polymers, and/or pharmacomodulation by chemical modifications of the peptide sequence) against the degradation by proteases of the gastrointestinal system, serum, kidney and liver (68, 69, 71, 72), ii/ the ever-increasing number of multi-resistant bacterial strains against commonly used antibiotics, like penicillin, streptomycin, vancomycin and fluoroquinolones, along with the depletion of antibiotic pipelines, and a worldwide resurgence of infectious diseases (73-75), iii/a more flexible stance of the US FDA with respect to AMPs illustrated by the launching in 2012 of the Antibacterial Drug Development Task Force in charge of facilitating “the use of innovative trial designs and alternative measures of clinical effectiveness”. A similar attitude was adopted in 2012 by the European Medicines Agency which has published new guidance documents outlining the clinical criteria for evaluating antimicrobials (70), and iv/the finding that AMPs are multi-functional host-defense peptides, paving the way for new therapeutic applications (76).

Table 5. Patents related to temporins[1]

Title	Web link[2]
Activatable membrane-interacting peptides and methods of use	WO2014160037 (A2)
Analogs of temporin-SHa, and uses thereof	WO2010106293 (A1)
Analogues of temporin-SHa and uses thereof	WO2015044356 (A1)
Anti-HIV peptides and methods of use thereof	WO2011049914 (A2)
Anti-microbial peptides and methods of use thereof	WO2013078217 (A2)
Antibacterial peptides from hylarana guentheri and application of antibacterial peptides	CN103965340 (A)
Anticancer treatment	WO2014003537 (A1)
Antimicrobial fusion compounds and uses thereof	WO2012093931 (A1)
Antimicrobial peptides isolated from the skin of American frogs	WO0009553 (A2)
Antimicrobial water treatment membranes and production thereof	WO2011070573 (A1)
Antimicrobially active polypeptides	WO9825961 (A1)
Bloody noun antibacterial peptide temporin-La, genes thereof and use in pharmacy	CN101475630 (A)
Bloody noun antibacterial peptide temporin-Lb, genes thereof and use in pharmacy	CN101503459 (A)
Matrix compositions for controlled release of peptide and polypeptide molecules	US2014271861 (A1)
Method for expressing temporin-1Sd and cecropin B2	CN103290055 (A)
Methods and compositions for controlling rotifers	US2014296137 (A1)
Novel antibacterial peptide and preparation method and use thereof	CN101418039 (B)
Pathogen inducible plant promoter and use thereof	CA2469098 (A1)
Peptide turn mimetics	US2009275727 (A1)
Production method of genetic engineering hybrid antimicrobial peptide CecA-Temporin-SHF	CN102952820 (A)
Silkworm bioreactor for producing fodder antimicrobial peptides and construction method thereof	CN103421844 (A)
Transgenic plants expressing temporin peptides	US7081568 (B2)
Transgenic plants that are resistant to a broad spectrum of pathogens	WO0055337 (A1)

[1]: Patents were obtained after a search in Google patent (https://www.google.fr/advanced_patent_search?hl=fr) and Espacenet (http://worldwide.espacenet.com/advancedSearch?locale=en_EP).
[2]: Espacenet link of each patent

A number of them trigger and tune immunomodulatory processes on host cells, by inducing chemotaxis, leukocyte activation and cytokine release, wound repair, and enhancing angiogenesis. Some peptides also act on a variety of pathogens, including fungi and protozoa that are the causative agents of malaria, leishmaniasis, amebiasis, and Chagas disease (77). Certain AMPs have potent antiviral activity (35, 78), others show selective cytotoxicity against mammalian tumor cells (79). Last, there is now a widespread acceptance that several antimicrobial peptides may also affect microbial viability by interactions with intracellular targets or disruption of key intracellular processes (14). Consequently, biotech and pharmaceutical companies are turning back to the commercial development of antimicrobial agents as illustrated by a rise in patent applications and R&D work. Ten antimicrobial peptides are actually in development pipelines or in clinical trials, including pexiganan again (phase III), which is now developed by Dipexium Pharma/ MacroChem/Genaera (70).

Frog skin is by far the most abundant source of animal AMPs, with 1007 peptides on 2001 listed in the Antimicrobial Peptide Database (last update: January 29, 2016) (Figure 1). Many frog skin AMPs or derivatives show very high potency against antibiotic-resistant bacteria and protozoa and have potential as lead compounds for new antibiotics. Examples of promising candidates include (E4K)alyteserin-1c, from *Alytes obstetricans*, active against colistin-resistant strains of multidrug-resistant *Acinetobacter baumannii* (80), the caerulein-precursor fragment CPF-SE3, from *Silurana epitropicalis,* active against clinical isolates of methicillin-resistant *Staphylococcus aureus* (81), and (E6k, D9k)hymenochirin-1B, from *Hymenochirus boettgeri,* active against a range of metallo-beta-lactamase-carbapenemase-producing clinical isolates of Gram-negative bacteria (82). Since most of the frog skin AMPs are multifunctional, alternative therapeutic applications as anti-cancer, anti-viral, anti-inflammatory, immunosuppressive, immunostimulatory, and anti-diabetic agents are also been actively explored (see (83) for an authoritative review) in addition to their potential development as antimicrobials. However, efforts to obtain a new generation of drugs using these peptides or mimetic compounds are still challenging with high associated R&D costs because of their large size (up to 48 residues), cytotoxicity, and the frequent presence of post-translational modifications (C-terminal amidation, pyroglutamylation, disulfide bridges). Because of these difficulties, the recent trend is to look for very short linear peptide sequence (naturally occurring frog skin AMPs or truncated analogues of long peptides) that favors cost-efficient chemical synthesis. Such short sequences are also easily amenable to optimization of the structure and to solution-phase synthesis that offer scalability advantages and reduced costs over solid-phase chemistry. Herein

Table 6. Temporins and temporin analogs with potential as lead compounds for new antibiotics

Peptide	Amino acid sequence[1]
Temporin-Ta	FLPLIGRVLSGIL$_a$[2]
Temporin-Tb	LLPIVGNLLKSLL$_a$
Temporin-TI	FVQWFSKFLGRIL$_a$
(Pro^3) temporin-TI	FV**P**WFSKFLGRIL$_a$
(Pro^3, D-Leu^9) temporin-TI	FV**P**WFSKF**dL**GRIL$_a$
Temporin-DRa	HFLGTLVNLAKKIL$_a$
(D-Lys^5) temporin-DRa	HFLG**dK**LVNLAKKIL$_a$
(D-Lys^8) temporin-DRa	HFLGTLV**dK**LAKKIL$_a$
(Aib^{13}) temporin-DRa	HFLGTLVNLAKK **(AibL)**$_a$
Temporin-SHa	FLSGIVGMLGKLF$_a$
(Lys^3) temporin-SHa	FL**K**GIVGMLGKLF$_a$
Temporin-SHf	FFFLSRIF$_a$
(p-^{t}BuPhe^2, Arg^5) temporin-SHf	F **(p-^{t}BuF)** FLRRIF$_a$

[1]: Modified amino acid residues are in bold (d: residue in D-configuration; AibL: α-aminoisobutyryl-L; p-tBuF: 4-tert-butyl-F), [2]:a=C-terminal amidation

we highlight recent advances in R&D regarding the temporins, which are among the shortest natural AMPs found in the frog skin, with 8-17 residues. Several patents related to temporins are already available, emphasizing the commercial and therapeutic values of these peptides. Using Google patent (https://www.google.fr/advanced_patent_search?hl=fr) and Espacenet (http://worldwide.espacenet.com/advancedSearch?locale=en_EP), we have listed these patents in Table 5. They provide valuable information on the potential applications of temporins.

4.1. Temporins and temporin analogs with therapeutic potential as antibacterial agent

Temporins represent a prototypical minimal model for membrane-destabilizing peptides with various selectivity as well as attractive templates for the design of new therapeutic agents against microbial pathogens. In this chapter, we will focus on a few well-characterized members having promising therapeutic and commercial applications and originating from Eurasian, North American and North African ranid frogs (Table 6).

As stated above, several members of the temporin family display very high efficiency *in vitro* against Gram-positive bacteria, including clinical isolates of methicillin-resistant staphylococci and antibiotic-resistant enterococci (10, 39, 46). Internalization and intracellular accumulation of *Staphylococcus aureus* in keratinocytes is a characteristic feature of several inflammatory skin diseases, as well as in those with skin lesions due to wounds or inserted medical devices, which is often followed by tissue invasion and severe cell damage. *S. aureus* can also become more invasive and cause life-threatening infections such as bacteremia, pneumonia, meningitis, osteomyelitis, endocarditis, and sepsis. Recent experiments have shown that temporin-Ta, FLPLIGRVLSGIL$_{amide}$, and temporin-Tb, LLPIVGNLLKSLL$_{amide}$, from *R. temporaria* are able to enter human immortalized keratinocytes (HaCaT cells) infected by MDR clinical isolates of *Staphylococcus* and to kill within 30 min the intracellular pathogen without affecting the viability of the infected cells (84). The precise mechanism of action subtending the killing activity of the internalized peptides towards the entrapped microbial pathogens is yet unknown, although co-localization of the peptides and the bacteria suggests a direct effect on the bacterial membrane. Furthermore, both temporins promote the closure of a pseudo-wound produced in a keratinocyte monolayer of HaCaT cells by a process involving the epidermal growth factor receptor signaling pathway (84).

Development of resistant bacteria onto implanted medical devices (catheters, prostheses, etc.) are responsible for over 60% of nosocomial infections, leading to clinical complications and death after surgery. Temporin-SHa, FLSGIVGMLGKLF$_{amide}$, has been grafted either via its NH2 groups or its C-terminal end on a gold surface modified by a thiolated self-assembled monolayer with carboxylic acid functionality (Figure 4). The efficacy and mode of action of the peptide-coated surface against the Gram-positive bacteria *Listeria ivanovii* was assessed by microscopy techniques, such as atomic force microscopy (AFM) and scanning electron microscopy equipped with a field emission gun (SEM-FEG) (85). Results showed that when this peptide is covalently grafted to the surface, the adhering bacteria are most probably permeabilized (killed or at least damaged), thus unable to grow, whereas bacteria integrity is not affected on a nonfunctionalized surface (Figure 4). Temporin-SHa therefore represents an attractive candidate as antimicrobial coating agent.

The therapeutic potential of many of the temporins as antibacterial agents is however limited by their weak activity against Gram-negative bacterial strains. The exceptions are temporin-TI, temporin-DRa, temporin-SHa, and temporin-SHd. Out of the earliest known temporins from *R. temporaria* (86), temporin-TI, FVQWFSKFLGRIL$_{amide}$, which bears a net positive charge of +3, is the most promising being highly active against clinically relevant Gram-negative bacteria such as *E. coli*, *Acinetobacter baumannii* and *A. junii*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, and *Yersinia pseudotuberculosis* with MICs ranging from 3 to 24 μM (45). Recent work of Mangoni and coworkers (87, 88) described the molecular basis for the low versus high potencies of temporin-Ta, -Tb

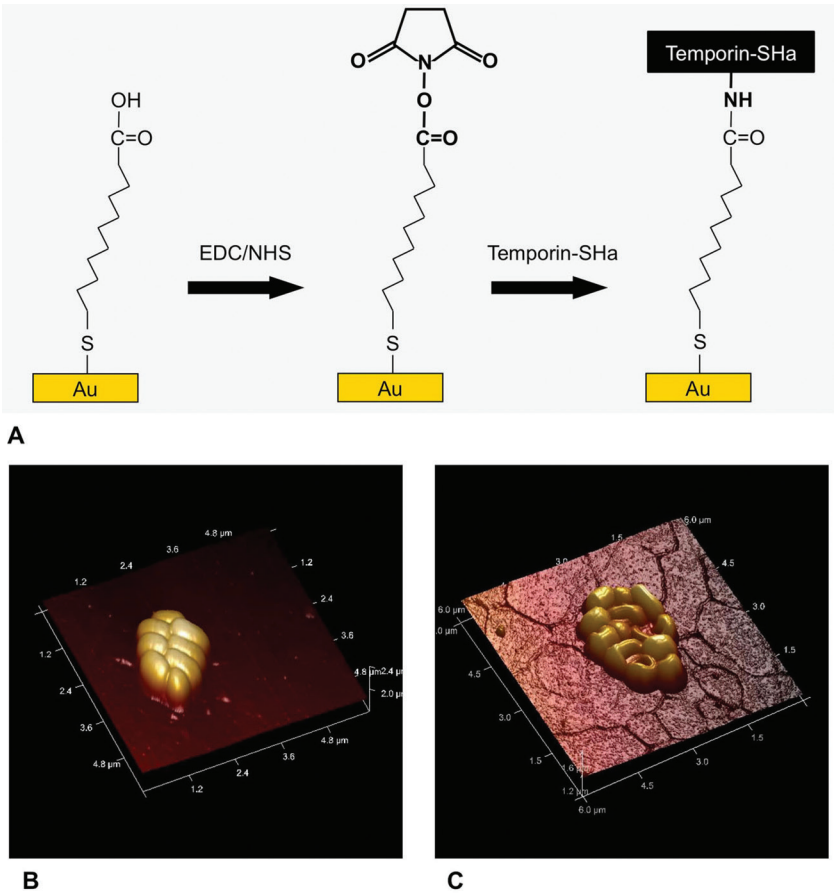
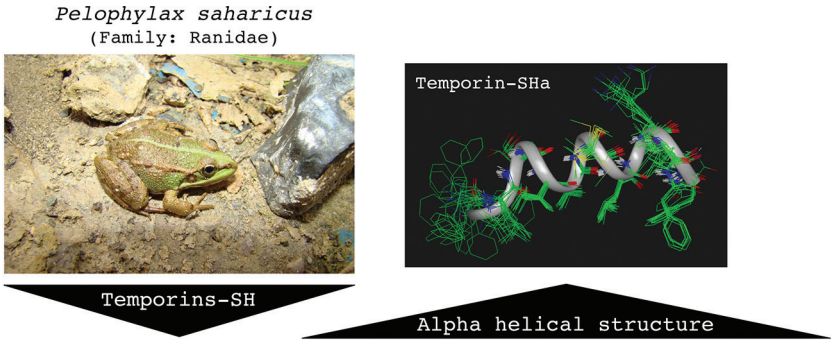


Figure 4. Anti-*Listeria ivanovii* activity of temporin-SHa grafted on a gold surface modified by a thiolated self-assembled monolayer (SAM) with carboxylic acid functionality. A) Covalent grafting of temporin-SHa. The acidic groups of the AU-MUA surface were activated for 60 minutes with a solution containing NHS (20 mM) and EDC (10 mM). Temporin-SHa (5 µg/mL) was then added onto the activated surface during 2 hours in order to ensure the covalent binding with the peptide amino groups. B) AFM images of *Listeria ivanovii* on Au-MUA surface without temporin-SHa (negative control). Alive and well-shaped bacteria are observed. C) AFM images of bacteria deposited on Au-MUA-temporin-SHa surface. Adhering bacteria are damaged (permeabilized or killed) by the grafted temporin-SHa, as indicated by the morphological changes. AFM images were provided by our collaborator, Dr. Vincent Humblot (Laboratoire de Réactivité de Surface, UMR 7197 UPMC-CNRS, Paris, France).

and -TI against Gram-negative bacteria. The highly anionic and compact lipopolysaccharide (LPS)-outer membrane of Gram-negative bacteria is known to act as a barrier against cationic AMPs. In order to disrupt the outer membrane through displacement of Mg^{2+} cations stabilizing adjacent LPS molecules, AMPs should acquire a specific structure in LPS (89). In the presence of LPS micelles, temporin-Ta and temporin-Tb undergo aggregation mediated by contacts among hydrophobic residues located at the N- and C-termini of the peptides, thus preventing their translocation to the cytoplasmic membrane. In contrast, temporin-TI assumes a basket-shaped amphipathic dimeric structure in LPS micelles, which favors a disaggregation of LPS micelles via, ionic and/or hydrogen-bonding interactions of the side chain of Arg^{11} from each helical subunit with the bisphosphate groups of lipid A (90, 91). Hence, temporin-TI penetrates efficiently into LPS monolayers to reach and permeate the cytoplasmic membrane of the bacteria. Synergistic effects in inhibiting the growth of Gram-negative bacteria and in neutralizing the toxic effect of LPS have been found when temporin-TI is combined either with temporin-Ta or -Tb (87, 88). It was proposed that the dimeric temporin-TI, once bound to LPS, prevents the aggregation of temporin-Ta or -Tb through changes in the structural states of LPS, hence allowing both peptides to reach the cytoplasmic membrane (87, 88).

On the negative side, however, temporin-TI is strongly hemolytic at microbicidal concentrations, which severely limits its clinical utility. In order to reduce the cytotoxicity of the peptide without affecting its antibacterial activity, a library of derivatives was designed and screened focusing on the correlation between the alpha-helix content of the peptides, the nature and position of their cationic residues, and their antibacterial and hemolytic activities (45). The percent helicity of analogs correlates directly with their hemolytic activities



Name	Sequence	Residue	Net charge
Temporin-SHa	FLSGIVGMLGKLF$_{amide}$	13	+2
Temporin-SHb	FLPIVTNLLSGLL$_{amide}$	13	+1
Temporin-SHc	FLSHIAGFLSNLF$_{amide}$	13	+1
Temporin-SHd	FLPAALAGIGGILGKLF$_{amide}$	17	+2
Temporin-SHe	FLPALAGIAGLLGKIF$_{amide}$	16	+2
Temporin-SHf	FFFLSRIF$_{amide}$	8	+2

Figure 5. Temporins-SH from *Pelophylax saharicus*. Six temporin members, named temporin-SHa, -SHb, -SHc, -SHd, -SHe and -SHf were identified from the skin of *Pelophylax saharicus* (family Ranidae). They differ in size (from 8 to 17 residues), amino acid sequence and net charge. As shown for temporin-SHa, temporins-SH forms alpha-helices (three dimensional structure obtained from (39)). The picture of *Pelophylax saharicus* was kindly provided by Radek Sejkora.

but not with their antibacterial activities, a finding in lines with previous studies demonstrating correlation between the extent of helical structure and toxicity of linear AMPs. The analog (Pro^3)temporin-TI displayed slightly lower antibacterial activity than temporin-TI but more than half of the toxic effect of the parent peptide. Replacing single amino acids within the helical domain of the peptide with the corresponding D-enantiomers (helix breakers) led to identification of (Pro^3, D-Leu^9)temporin-TI as a highly potent peptide practically devoid of toxicity towards human erythrocytes and keratinocytes (92). In addition, incorporation of a D-amino acid within the peptide sequence should increase its resistance to proteolysis *in vivo*. Thus, breaking the helicity of temporin-TI via the substitution of Gln^3 with Pro and replacement of Leu^9 with its D-enantiomer play a key role in distinguishing between prokaryotic and eukaryotic membranes. These findings identify (Pro^3, D-Leu^9) temporin-TI as a small-sized peptide with high potential for development as a therapeutic valuable anti-infective agent.

The tetradecapeptide temporin-DRa, HFLGTLVNLAKKIL$_{amide}$, from *Rana draytonii*, which bears a positive charge of + 3 at neutral pH, is highly potent against clinical isolates of MRSA (MIC ~ 8 µM) and displays growth-inhibitory activity against the Gram-negative bacteria *E. coli*, *Klebsiella pneumoniae*, *Enterobacter cloacae*, *Enterococcus faecalis* *Pseudomonas aeruginosa*, and *Salmonella typhimurium*, with MICs ranging from 12 to 25 µM (93). The peptide is appreciably hemolytic against human erythrocytes (LC_{50} ~ 70 µM) giving low therapeutic indices (LC_{50}/MIC ~ 3 to 6). Analogs of temporin-DRa in which each amino acid was replaced by Lysine or D-Lysine were screened against a range of Gram-positive and Gram-negative bacteria (46). In accordance with several previous studies with model alpha-helical AMPs demonstrating positive correlations between cationicity and antibacterial activity and between helicity/amphipaticity and hemolytic activity, the analogs (Lys^7)temporin-DRa, (D-Lys^5)temporin-DRa, and (D-Lys^8)temporin-DRa retain high activities against MRSA an Gram-negative bacteria (MICs in the range 8-16 µM) but have very low hemolytic activities (LC_{50} > 330 µM) (94). Effects of conformational constraints on the activity of temporin-DRa were also evaluated through the incorporation of alpha-aminoisobutyric acid (Aib) into the peptide sequence, which restraints the rotation of the torsional angles around the alpha carbon (94). Incorporation of Aib into a linear peptide either promotes the formation of an alpha- or 3_{10}-helix or stabilizes the existing helical conformation (95). Substitutions at Leu^9, Ile^{13}, and Leu^{14} by Aib generate analogs with decreased hydrophobicity and helicity that have potencies against Gram-positive and Gram-negative bacteria identical to those of the parent peptide but show much lower toxicity against mammalian cells. Among these analogs, (Aib^{13})temporin-DRa with a fourfold increase in therapeutic index (LC_{50}/MIC = 20) compared to the parent molecule appears as a compound with potential for R&D as a therapeutically valuable antibacterial agent.

Temporins from *Pelophylax saharicus* differ widely in size (from 8 to 17 residues), net charge and antibacterial activity (Figure 5). Temporin-SHa, FLSGIVGMLGKLF$_{amide}$, is the first 13-residue member

of the temporin family with a net charge of + 2 that exhibits a broad spectrum of antimicrobial activity, being cidal against Gram-positive and Gram-negative bacteria, with particularly high potency on the clinically relevant Gram-negative strains *Escherichia coli* (MIC = 10 μM) and *Pseudomonas aeruginosa* (MIC = 30 μM) (40, 43). However, this peptide displays significant hemolytic activity against human erythrocytes above the MIC values observed for most of the bacterial strains tested (LC_{50} = 25 μM). The simple replacement of the serine residue at position 3 by a lysine led to an analog, (Lys^3) temporin-SHa, that retained the high potency of the parent peptide against Gram-positive bacteria but was however 2- to 8-fold more efficient than temporin-SHa in killing Gram-negative bacteria, including *E. coli* , *P. aeruginosa*, *S. enterica*, *A. baumannii*, and *K. pneumoniae* (MIC = 3 - 6 μM) (unpublished results). At antibacterial concentrations (1 - 6 μM), no cytotoxicity was observed for (Lys^3)temporin-SHa toward human erythrocytes, THP-1 monocytes and THP-1-derived macrophages, HepG2 cells and fibroblasts (LC_{50} = 50 - 360 μM). Multipassage resistance selection experiments using (Lys^3)temporin-SHa and *E. coli* for resistance testing showed that the analog display no propensity to select bacterial resistance compared to ampicillin Therefore, (Lys^3)temporin-SHa emerged as a highly potent and non-cytotoxic analog having a wide spectrum of activity against Gram-negative and Gram-positive bacteria.

Although temporin-SHf, $FFFLSRIF_{amide}$, has moderate sterilizing activity toward Gram-positive (MIC = 3 - 50 μM) and Gram-negative (MIC = 25 - 100 μM) bacteria (42), its short length, compositional simplicity and harmlessness with respect to mammalian cells make it a promising model compound for the rational development of new antibacterial agents. Despite the fact that the peptide chain of temporin-SHf is very short, its mode of antimicrobial action involves binding and penetration into the bilayer, causing disruption of acyl chain packing, efflux of large molecules and microbial cell death, similar to that of many long AMPs. Considering the interesting characteristics of temporin-SHf, a series of derivatives containing insertion of a basic arginine residue, as well as residues containing neutral hydrophilic (serine and alpha-hydroxymethylserine) and hydrophobic (alpha-methyl phenylalanine and *p*-^{t}butyl phenylalanine) groups, were designed with the aim to improve its antimicrobial efficiency while retaining its nonhemolytic character (96). Among these analogs, (*p*-^{t}BuPhe^2, Arg^5) temporin-SHf exhibited a remarkable increase of potency against a wide range of clinically interesting Gram-positive (MIC = 3 - 25 μM) and Gram-negative bacteria (MIC = 6 - 12.5. μM) compared to the parent peptide. Interestingly, (*p*-^{t}BuF^2, Arg^5)temporin-SHf is also active at physiological salt concentrations and in 30% serum. This study indicates that both natural and unnatural amino acids can be used to produce ultrashort and highly potent AMPs with broad antibacterial spectrum.

4.2. Temporins with therapeutic potential as antiparasitic agents

Despite their small size and low cationicity, some temporins (Ta, Tb, Tf, Tl, SHa and SHd) are also able to kill the human parasitic protozoan *Leishmania*, the causative agent of leishmaniasis, a neglected tropical disease endemic in over 80 countries worldwide leading to morbidity and mortality (97). More than twenty *Leishmania* species are transmitted to humans by the bites of infected phlebotomine sandflies (insect vector) and cause either cutaneous leishmaniasis (severe ulcerative lesions), mucocutaneous leishmaniasis (partial or total mutilation of mucous membranes of the nose, mouth and throat), or visceral leishmaniasis, which is fatal if left untreated. Although leishmaniasis is one of the major parasitic diseases in the world, its chemotherapeutic treatment is today unsatisfactory and relies mainly on pentavalent antimonials that are highly toxic, require daily injections, and whose efficacy is threatened by increasing resistance. *Leishmania* is an obligate intracellular protozoan parasite of mononuclear phagocytes *in vivo*. Its life cycle consists of two stages: an extracellular promastigote form that can be found in the gut of the insect vector, and an intracellular non-motile amastigote form that occurs in the vertebrate host within the parasitophorus vacuole of the macrophages. The intracellular location of the parasite protects the microorganism from many of the usual host defense mechanisms. Interestingly, some temporins were demonstrated to be active on both promastigotes (insect stage) and amastigotes (mammalian stage). Although, the antiparasitic mechanism of temporins remains unclear, particularly the molecular/cellular mechanisms by which temporins kills amastigotes in the macrophages, some data are available on their leishmanicidal activity and on the membrane components of *Leishmania* promoting susceptibility to temporins (49-51).

Temporin-Ta and -Tb were first reported to show potent activity against *Leishmania*. These temporins demonstrated killing activity (LC_{50} = 15-25 μM) against *L. donovani* promastigotes and *L. pifanoi* axenic amastigotes (extracellular amastigote maintained in culture). Both peptides preserve their function at physiological salt concentration and in 33% human serum (49).

Two temporins from *Pelophylax saharicus*, temporin-SHa and temporin-SHd, exhibit also a significant leishmanicidal activity against *L. infantum* responsible, like *L. donovani*, of visceral leishmaniasis. Temporin-SHa kills *L. infantum* promastigotes and axenic amastigotes with an IC_{50} of 18 μM and 22 μM, respectively (43), but also intramacrophagic amastigotes, the more resistant mammalian intracellular stage (unpublished results). At doses that are lethal for the parasite, the peptide has no harmful effect on the macrophages. Interestingly, temporin-SHa retains ability to kill with the same efficiency *L. infantum* parasites and antimony-resistant

L. infantum. Moreover, this peptide is active against other *Leishmania* species, such as *L. major* and *L. amazonensis* (responsible of cutaneous leismaniasis), *L. braziliensis* (mucocutaneous leismaniasis), and also against other parasites of the Trypanosomatidae family, such as *Trypanosoma*. The 17-residue long peptide, temporin-SHd, showed potent activity against *L. infantum* promastigotes and axenic amastigotes with an IC_{50} of 16.5 µM and 23.5 µM, respectively (51). The activity was 3-4 fold better (IC_{50} = 6.7 µM) against infected macrophages (intracellular amastigotes). This could be due to a penetration of the peptide into the macrophage and its accumulation within the parasitophorus vacuole combined to an immunomodulatory response of the macrophage. Like temporin-SHa, this peptide also displayed activities against other *Leishmania* species (*L. major*, *L. tropica*, and *L. amazonensis*: IC_{50} ~ 14 µM; *L. braziliensis*: IC_{50} = 18 µM), and against other Trypanosomatidae of the genus Trypanosoma (*T. brucei*: IC_{50} = 22 µM, *T. cruzi*: IC_{50} = 17 µM). Temporin-SHd is not toxic for human THP-1 monocytes (LC_{50} = 66 µM) and THP-1-derived macrophages (LC_{50} = 60 µM) at leishmanicidal concentrations (10-25 µM), and for human hepatoma-derived cells HepG2 up to 400 µM (51).

Using a set of different temporins, recent studies of Eggimann and collaborators have shown that *L. mexicana* amastigotes were more resistant to these peptides compared to promastigotes forms (50). For instance, temporin-Ta, -Tf, and -Tl, and temporin-SHa showed good activity against the insect stage of the parasite (ED_{50} = 4 - 14 µM) whereas *L. mexicana* amastigotes are resistant to temporin-Ta and -Tf (ED_{50} ≥ 100 µM), and show 10-fold and 17-fold lower susceptibility to temporin-SHa and temporin-Tl, respectively (50). The anionic proteophosphoglycan was shown to be a major factor of promastigote sensitivity to the temporins. Its absence at the surface of pathogenic *L. mexicana* amastigotes may be implicated in the resistance to these cationic peptides.

Considering the potent leishmanicidal activity of temporins, including resistant *Leishmania* parasites, and their ability to also kill efficiently the intracellular forms of the parasite without toxicity for the host macrophages, these small-size peptides are promising compounds for the development of a new class of antiparasitic agents with therapeutic potential.

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Abbreviations: AMP: antimicrobial peptide, LC50: concentration of peptide producing 50% hemolysis, ED_{50}: concentration of peptide causing 50% cell death, MIC: lowest concentration of peptide at which bacterial growth is completely inhibited, MDR: multi-drug-resistant, MRSA: methicillin-resistant *Staphylococcus aureus*. EDC: N-1-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride, NHS: hydroxysuccinimide, AU-MUA: gold-mercaptoundecanoic acid.

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