The Bioscan
AN INTERNATIONAL QUARTERLY JOURNAL OF LIFE SCIENCES
21(1): 808-818, 2026 www.thebioscan.com

Structural and Functional Characterization of Hejiangin-A1, a Potent Antimicrobial Peptide against Resistant Pathogens

D. Zarena

Department of Physics, JNTUA College of Engineering, Anantapur 515002, AP, India.
Email: zareenajntua@gmail.com

DOI: 10.63001/tbs.2026.v21.i01.pp808-818

Keywords
Hejiangin –A1, Antimicrobial peptide, Circular dichroism, NMR spectroscopy

Received on:

16-11-2025

Accepted on:

26-12-2025

Published on:

01-02-2026

ABSTRACT

Antimicrobial peptides (AMPs) are the potent components of host innate defense mechanism that protects organisms from biotic and abiotic environmental threats. By the course of evolution microbes acquired resistance to conventional antibiotics. In this context, AMPs provides an eminent source to develop new generation of antimicrobials to combat with antibiotic resistant microbes. In the present work, we selected a 16-residue peptide (RFIYMKGFGKPRFGKR), Hejiangin-A1 (APD ID: AP01891), a potent AMP from the skin secretions of *Odoranna hejiangensis*, a Chinese odorous frog which has not been characterized for its structure and mechanism (Yang X, et al., 2012). Different experiments like MIC of the peptide, hemolytic assay, circular dichroism and NMR spectroscopic studies in the presence of D8PG micelles were carried out to characterize the selected AMP, Hejiangin-A1. The AMP shown the activity against both gram^{+ve} and gram^{-ve} bacteria and human fungal strains and no hemolytic activity against the human red blood cells was found for the AMP. The CD spectrum of peptide, suggested an α-helical configuration in the LPS medium in 1:3 ratio at a pH of 7.4. Solution NMR studies of Hejiangin-A1 reveal that it does not adopt any structure in presence of D8PG micelles.

Introduction:

During the course of evolution most of the microbes including bacteria, fungi, protozoa etc., acquired resistance to conventional antibiotics constituting a severe threat to the human health and initiated the need for search of untherapeutic actions which helps to combat with these classical antibiotic resistant microbes (Norrby et al., 2005; Livermore, 2005). In this scenario antimicrobial peptides, constituents of host innate immunity emerged as potential antimicrobial agents from the last few years. Most of the multicellular organisms; insects, mammals, reptiles and plants produce antimicrobial peptides, which are potentially active against bacteria both gram positive and gram negative, fungi and viruses (Park et al., 2003; Boman et al., 1991; Boman, 1991). These AMPs protects host cell from the invading microbes by disrupting their

cell membrane and subsequent killing of the microbes and also reported to be less toxic to the host cells. (Wang et al., 2012; Mishra et al., 2017). More than 2800 naturally occurring AMPs from the six kingdoms (as of Dec 2017) were isolated and deposited in antimicrobial peptide database (https://aps.unmc.edu/) under different categories like antibacterial, antiviral, antiparasitic and antifungal etc., based on their activity.

AMPs are determined by their short sequences (ranging from 10 to 50 amino acids length), cationic nature (+2 to +9) and having an average hydrophobic content of 42% (Wang et al., 2016). These are often characterized by multiple number of amino acids like proline, arginine, lysine, tryptophan and phenylalanine and a high proportion (>30%) of hydrophobic amino-acids. Every species appears to produce its own set of AMPs depending on their surroundings against variety of microbes and other environmental factors for the survival (Amiche *et al.*, 1999; Conlon *et al.*, 2004). The main families of antimicrobial peptides belong to a large group of linear amphipathic helical peptides. These characteristics provide them with an ability to bind to negatively charged molecules and/or membrane lipids and disturb the membrane structure. This seems to be the main mechanism of induction of death of their targets (Nicolas and Mor, 1995; Simmaco *et al.*, 1998; Zasloff, 2002; Conlon *et al.*, 2004). The structural conformation of peptide plays a major role in disrupting bacterial membranes and disrupting the preformed biofilms *etc*. (Zarena et al., 2017).

Antimicrobial peptides were synthesized and stored in a specialized syncytial structures, granular glands or poison glands of the dermal layer which are surrounded by a layer of smooth muscle cells innervated by sympathetic nerves (Mills and Prum, 1984; Sjoberg and Flock, 1976). Antimicrobial peptides and other bioactive peptides are synthesized as larger proteins with a signal sequence and an acidic pro-piece that are cleaved to release the mature active peptide before or at the time of secretion from granular glands (Amiche *et al.*, 1999; Bowie *et al.*, 1999). Several researches are concentrating on the development of synthetic AMPs, structural design based on natural AMPs available on the AMP database and their activity in combination with naturally occurring AMPs against different microbes (Mishra et al., 2017).

Amphibians are the ancient creatures and are constantly exposed to the diverse environmental conditions, evolved with highly advanced innate immunity (Carey et al., 1999; Rollins-Smith

and Cohen, 2004). Amphibian skins, which are exposed directly to various survival conditions and can secret a remarkable array of AMPs, one of the components of innate immunity, aroused great attention in the past decades with a wide range of secreted antimicrobial peptides against invading pathogens, (Simmaco et al., 1998; Rollins-Smith, 2009; Conlon et al., 2004) and ability to permeabilize mammalian cells (Conlon, 2011). An extensive literature characterizes the amino acid sequences, nucleotide sequences, and activity of a large number of biologically active peptides isolated from amphibian skin (Erspamer, 1994). About 1049 active antimicrobial peptides from amphibians were documented in AMP database (http://aps.unmc.edu/AP/main.php), among those 980 were reported from the frog species (as of Dec 2017). By the discovery of magainins in the skin secretion of African clawed frog, *Xenopus laevis* (Zasloff, 1987; Giovannini et al., 1987), the research turned towards identification and characterization of skin secreted antimicrobial peptides from different frog species. In this context, the present work is carried out on the frog peptide, Hejiangin-A1.

Result and Discussion:

Antimicrobial and hemolytic assay:

Hejiangin-A1 showed activity against both Gram positive and negative bacterial strains as well as human fungal strains. It is more active against the Gram negative strains especially *P. aeruginosa*, a nosocomial pathogen containing an outer layer protective alginate capsule. Also the peptide did not had any haemolytic activity when tested against the human red blood cells (figure 1).

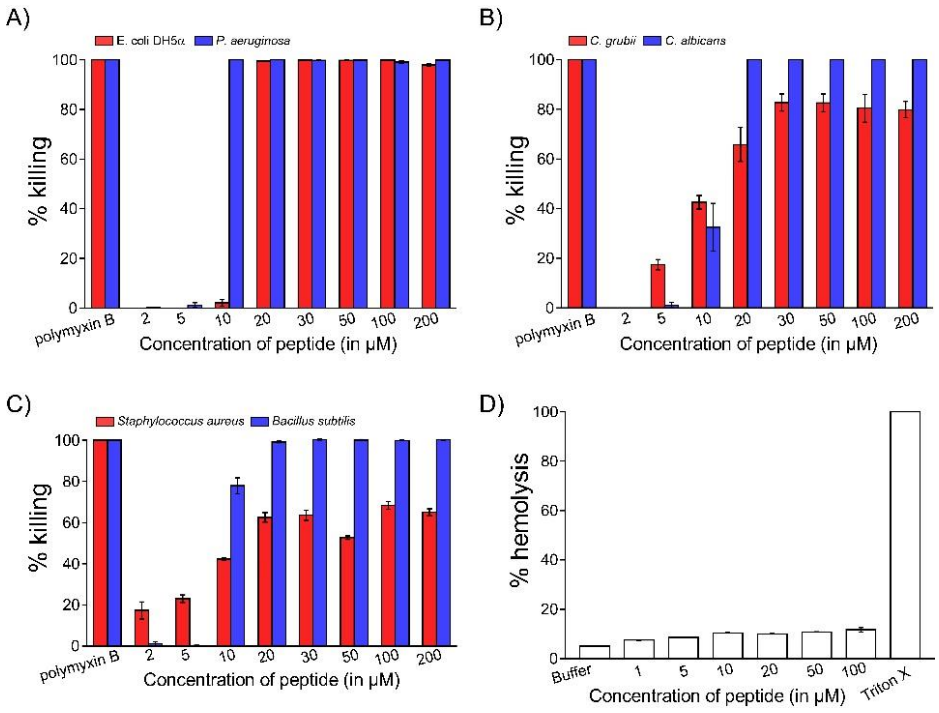

Figure.1: A, B and C: % killing of the bacterial and fungal strains by the peptide Hejiangin-A1 at various concentrations; D: hemolytic activity of Hejiangin-A1 at different concentrations.

Circular Dichroism studies:

Structural characterization of Hejiangin-A1 was done using circular dichroism and high resolution NMR studies. The CD spectra of the peptide (pH 7.4, 25ºC) in aqueous solution gave a negative band near 200nm. Addition of increasing concentration of LPS, above its CMC lead to changes in the CD profile thus indicating changes in structural characteristics. A slight positive maxima at 195 nm and two strong negative peaks, one at 208 nm and a lower intensity one at 222 nm, were observed, suggesting an α-helical conformation of the peptide in LPS under 1:3 ratio at pH 7.4 condition (figure 2). Deconvolution of the CD spectrum using CDNN software showed a helical content of ~42.5%, turn conformation of 19%, parallel and antiparallel β sheet conformation of 22% and 3%, respectively, and random coil conformation of ~7%. However, it should be noted that deconvolution with CDNN is only supplementary since the estimations made by the databases is based on globular proteins rather than linear amphipathic

peptides and may be skewed to some extent and was therefore done only for comparison purposes.

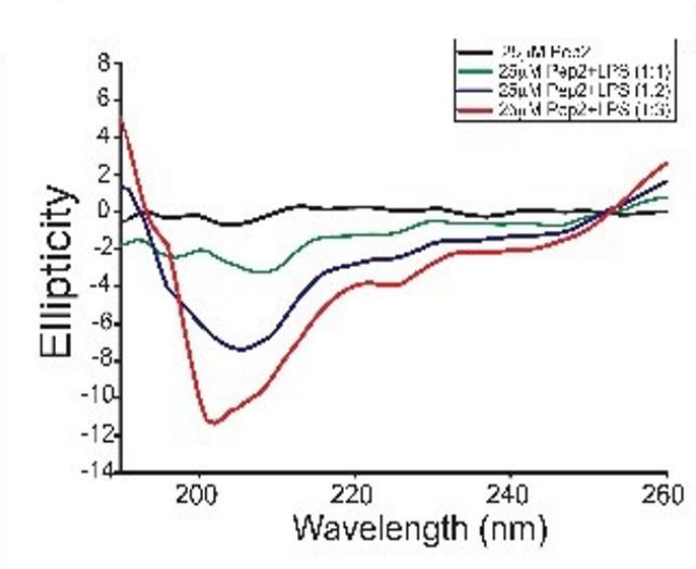

Figure.2: Circular dichroism of Hejiangin-A1

[1]H NMR studies:

[1]H NMR spectra of Hejiangin-A1 was recorded in absence and presence of 10mM D8PG, which act as a mimic for the bacterial membrane. Addition of D8PG to free peptide solution generated a well dispersed proton spectra, thus signifying the binding of the peptide to negatively charged membrane. This allowed us to further probe the D8PG bound functionally active conformation of peptide using two-dimensional NMR spectroscopy. The *tr*NOESY spectra of peptide in D8PG micelle exhibited the presence of sequential αN (i, i+1) as well as NH/NH (i, i+1) NOEs for all the residues. The absence of medium (i, i+2/i+3), side chain/ side chain or side chain/NH and long range (i, ≥i+5) NOEs for residues in both N- and C- termini confirms that it adopts a random coil/turn structure in SDS micelle. Due to lack of signature NOEs, it was difficult to determine the exact secondary structure assumed by the peptide in micellar environment. The

peptide 3.A) Hejiangin-A1 hence not adopts any known conformation in association with D8PG micelles (figure 3A and 3B).

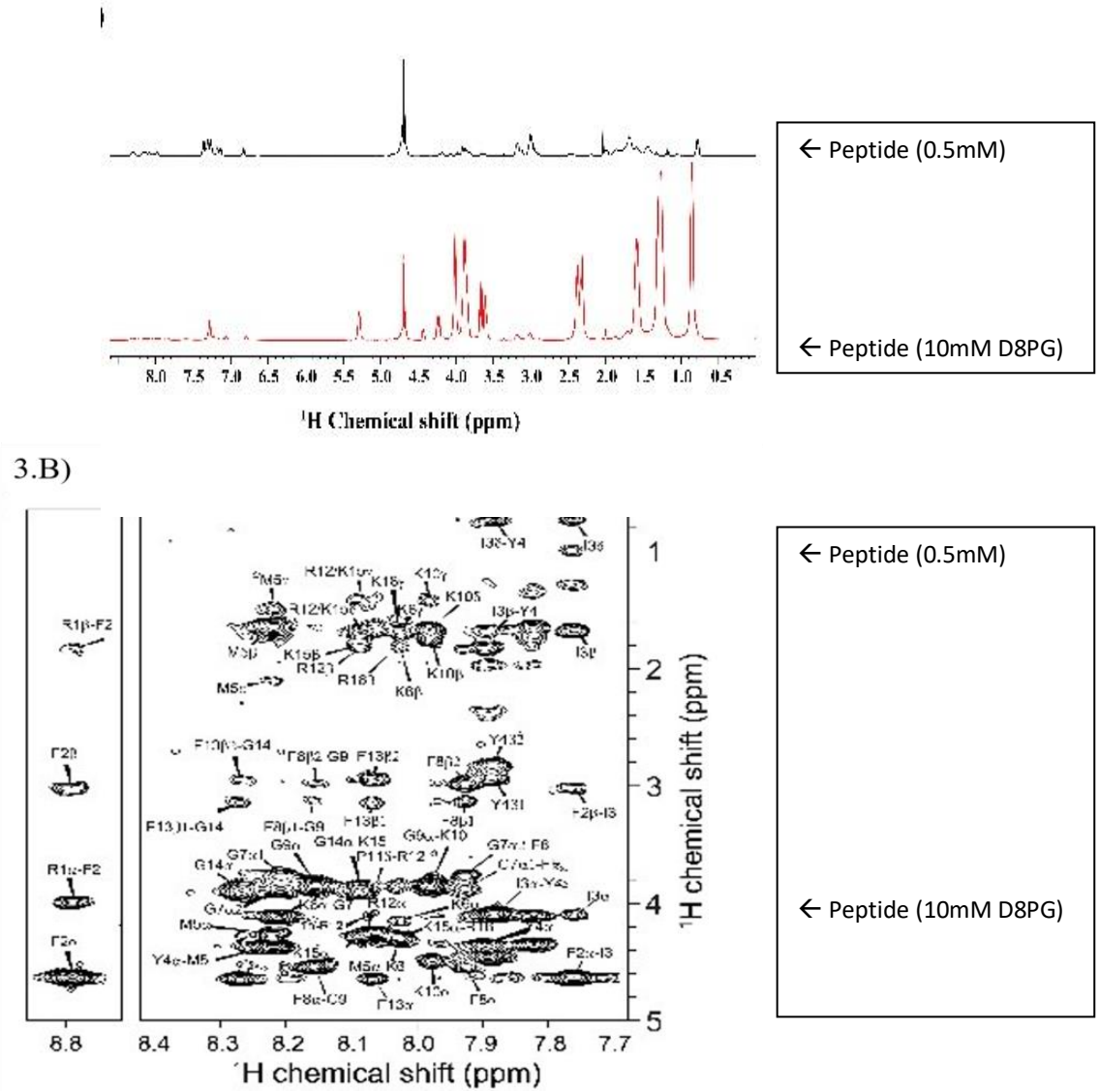

Figure. 3A and 3B: ^{1}H NMR spectrum of Hejiangin-A1

Materials and methods:

Microbroth dilution assay:

A modified version of the standard microdilution broth assay as described previously, was used to evaluate antimicrobial activity of the peptides (PMID: 11352918). Overnight grown cultures

of the respective pathogens were used to obtain the mid log phase cultures of *E. coli* DH5α, *P.aeruginosa*, *B.subtilis*, *S.aureus*, *C.albicans* and *C.grubii*. The cell suspensions were centrifuged at 6000 rpm for 5 minutes and washed thrice with 10 mM phosphate buffer of pH 7.4 and finally re-suspended in the same buffer to obtain a cell suspension containing 10^5 CFU/mL. The reaction was set in a 96 well plate, 50µL of the cell suspension was incubated with different concentrations of peptide (ranging from 1µM to 100µM) using a 1mM peptide stock in sterilized millipore water and incubated at their respective temperature (37°C for bacterial and 28°C for fungal culture) for 4 hours with shaking. A negative control containing only cell suspension and a positive control containing 10µM Polymyxin B with cell suspension was maintained. 180µL of suitable media was added to each well and incubated overnight with shaking at the respective temperature. Absorbance of the culture was read at 630nm to monitor bacterial growth. The positive control Polymyxin B was used to normalize all other readings. "The peptide concentration at which 99% growth inhibition was observed served as its $MIC_{99\%}$" (minimum inhibitory concentration at which 99% microbial cells are killed). All experiments were performed in triplicates.

Hemolytic assay:

Fresh human blood collected in EDTA vial was used for hemolytic activity analysis. Erythrocytes pellet were obtained by centrifugation at 8000g for 10min at 4°C and was washed thrice with PBS (pH 7.4) followed by final re-suspension in the same buffer to obtain a 2×10^8cells/mL suspension. Cells were further incubated with equal volumes of increasing concentrations of the peptide up to 100µM at 37°C under shaking for 1 hour. Samples were again centrifuged at 8000 g for 10min at 4°C and the absorbance of supernatant was measured at 414nm to quantify RBC lysis and heme release. Heme released by 2% Triton X 100 was taken as 100% and data from other samples were normalized against it. All experiments were performed in triplicates.

Circular Dichroism Study:

The secondary structure of the peptide was studied in solution as well as in the presence of LPS using a Jasco 815 spectrometer. Both peptide and LPS stocks were prepared in 10mM phosphate buffer, pH 7.4. 25µM peptide was titrated with increasing concentrations of LPS, ranging

between 25 to 75µM. The spectra were recorded at room temperature (25ºC) with accumulations of three scans at a speed of 100nm/min using a 0.1cm path length cuvette. Spectra were acquired from 190-260nm, with a data interval of 1nm and were corrected using a baseline spectrum in each case. The ellipticity in millidegrees was plotted against wavelength in nm.

NMR Spectroscopy:

NMR experiments were carried out using Bruker Avance III 500MHz NMR spectrometer, equipped with 5mm SMART probe at 310K. The NMR sample was prepared in 10% deuterated water (pH 4.5) and DSS (2, 2-Dimethyl-2-silapentane-5- sulfonate sodium salt) was used as an internal chemical shift standard. 1D proton NMR spectra of 0.5mM peptide, was recorded alone and in the presence of dioctanoyl phosphatidylglycerol (D8PG) micelle at a ratio of 1:10. Water suppression was done using Excitation-sculpting scheme and the States-TPPI for quadrature detection in the t1 dimension (https://doi.org/10.1006/jmra.1993.1098). Subsequently, two-dimensional ^{1}H-^{1}H total correlation spectroscopy (2D TOCSY), and two-dimensional ^{1}H-^{1}H Nuclear Overhauser Spectroscopy (2D NOESY) was recorded with a mixing time of 80ms and 150ms respectively and spectral width of 14ppm in both directions. The number of scans was fixed at 28 and 96 respectively per t1 increment with 16 dummy scans.

NMR data processing, and analysis, was performed using Topspin (Bruker) and SPARKY (www.cgl.ucsf.edu/home/sparky/) software, respectively. Sequence, specific resonance assignment, was performed based on 2D TOCSY and NOESY peaks using standard assignment techniques.

Author information: D. Zarena and Late Prof. H.S. Atreya, NMR Research Centre, Indian Institute of Science, Bangalore designed the project and directed the research. DZ thank Humaira Ilyas for performing experiments and Dr. Anirban Bhunia, Bose institute, Kolkata for his generous support in providing lab facilities.

Acknowledgement

This work was supported by the grant from DST-SERB, New Delhi (F.No. SB/SO/BB-0014/2013 dated 17/10/2013) awarded to Dr. D. Zarena.

References:

1. Norrby SR, Nord CE, Finch R. Lack of development of new antimicrobial drugs: a potential serious threat to public health. Lancet Infect Dis 2005; 5: 115-9.

2. Livermore DM. Has the era of untreatable infections arrived? J Antimicrob Chemother 2009; 64 Suppl 1: i29-36.

3. Park, Y. et al. "A Leu-Lys-rich antimicrobial peptide: activity and mechanism". Biochimica et Biophysica Acta 1645, 172-182 (2003).

4. Boman, H. G., Faye, I., Gudmundsson, G. H., Lee, J.-Y. & Lidholm, D.-A. "Cell-free immunity in Cecropia. A model system for antibacterial proteins". European Journal of Biochemistry 201, 23-31 (1991).

5. Boman, H. G. "Antibacterial peptides: key components needed in immunity". Cell 65, 205-207 (1991).

6. Carey, C., N. Cohen, and L. Rollins-Smith. 1999. Amphibian declines: An immunological perspective. Dev. Comp. Immunol. 23:459–472.

7. Rollins-Smith, L. A. and N. Cohen. 2004. Hormones and the immune system of amphibians. In H. Heatwole (ed.), Amphibian biology, Vol. 6, Endocrinology, pp. 2377–2391. Surrey Beatty and Sons, Chipping Norton, Australia. (In press).

8. Davidson, C.; et al. Effects of chytrid and carbaryl exposure on survival, growth and skin peptide defenses in foothill yellow-legged frogs. Environ. Sci. Technol. 2007, 41, 1771–1776.

9. Simmaco, M., G. Mignogna, and D. Barra. 1998. Antimicrobial peptides from amphibian skin: What do they tell us? Biopolymers (Peptide Science) 47:435–450.

10. Conlon, J. M., J. Kolodziejek, N. Nowotny. 2004. Antimicrobial peptides from ranid frogs: Taxonomic and phylogenetic markers and a potential source of new therapeutic agents. Biochim. Biophys. Acta. 1696:1–14.

11. Rollins-Smith, L. A. (2009). The role of amphibian antimicrobial peptides in protection of amphibians from pathogens linked to global amphibian declines. Biochim.Biophys. Acta 1788, 1593-1599.

12. Conlon JM. The contribution of skin antimicrobial peptides to the system of innate immunity in anurans. Cell Tissue Res 2011; 343: 201-12.

13. Wang, G., Epand, R.F., Mishra, B., Lushnikova, T., Thomas, V.C., Bayles, K.W. and Epand, R.M., 2012. Decoding the functional roles of cationic side chains of the major antimicrobial region of human cathelicidin LL-37. Antimicrobial agents and chemotherapy, 56(2), pp.845-856.

14. Mishra B, Lushnikova T, Golla RM, Wang X, Wang G: Design and surface immobilization of short anti-biofilm peptides. Acta Biomater. 2017, 49:316-328.

15. Zasloff M. Magainins, a class of antimicrobial peptides from Xenopus skin: isolation, characterization of two active forms and partial cDNA sequence of a precursor. Proc Natl Acad Sci USA 1987; 84: 5449-53.

16. Mills, J. W. and B. E. Prum. 1984. Morphology of the exocrine glands of the frog skin. Amer. J. Anat. 171:91–106.

17. Sjoberg, E. and A. Flock. 1976. Innervation of skin glands in the frog. Cell Tiss. Res. 172:81–91.

18. Bowie, J. H., K. L. Wegener, B. C. S. Chia, P. A. Wabnitz, J. A. Carver, M. J. Tyler, and J. C. Wallace. 1999. Host defence antibacterial peptides from skin secretions of Australian amphibians. The relationship between structure and activity. Prot. Peptide. Lett. 6:259–269.

19. Zarena, D.; Mishra, B.; Lushnikova, T.; Wang, F.; Wang, G. The π Configuration of the WWW Motif of a Short Trp-rich Peptide Is Critical for Targeting Bacterial Membranes, Disrupting Preformed Biofilms and Killing Methicillin-resistant *Staphylococcus aureus*. *Biochemistry* 2017.

20. Giovannini MG, Poulter L, Gibson BW, Williams DH. Biosynthesis and degradation of peptides derived from *Xenopus laevis* pro hormones. Biochem J 1987; 243: 113-20.

21. Mishra, Biswajit, Scott Reiling, D. Zarena, and Guangshun Wang. "Host defense antimicrobial peptides as antibiotics: design and application strategies." *Current opinion in chemical biology* 38 (2017): 87-96.

22. Nicolas, P. and A. Mor. 1995. Peptides as weapons against microorganisms in the chemical defense system of vertebrates. Annu. Rev. Microbiol. 49:277–304.

23. Zasloff, M. 2002. Antimicrobial peptides of multicellular organisms.Nature 415:389–395.

24. Erspamer, V. 1994. Bioactive secretions of the amphibian integument. In H. Heatwole, G. T. Barthalmus, and A. Y. Heatwole (eds.), Amphibian biology, Vol. 1, The integument, pp. 178–350. Surrey Beatty and Sons, Chipping Norton.

25. Wang, G., Schmidt, C., Li, X. and Wang, Z. (2026) APD6: The antimicrobial peptide database is expanded to promote research and development by deploying an unprecedented information pipeline. Nucleic Acids Research 54, D363-D374.

26. Yang X, Lee WH, Zhang Y., Extremely Abundant Antimicrobial Peptides Existed in the Skins of Nine Kinds of Chinese Odorous Frogs *J. Proteome Res.*, 2012, *11* (1), pp 306–319

27. Amiche, M., A. S. Aurelia, N. P. Thierry, and P. Nicolas. 1999. The dermaseptin precursors: A protein family with a common preproregion and a variable C-terminal antimicrobial domain. FEBS Lett. 456:352–356.