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Antimicrobial peptides isolated from amphibian skin secretions: Structure and activity

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Abstract

It is widely observed that tropical frogs sit in warm, pathogen-rich environments yet rarely develop skin infections a paradox explained by the cocktail of antimicrobial peptides (AMPs) their granular glands secrete. This research isolated and characterised three cationic AMPs (designated KR-1, KR-2, and KR-3) from the skin secretions of the Chiang Mai stream frog (*Sylvirana nigrovittata*) collected in northern Thailand. Peptides were purified by gel filtration and reversed-phase HPLC, and their primary structures were determined by Edman degradation and confirmed by MALDI-TOF mass spectrometry. KR-1 (23 residues, MW 2,647 Da), KR-2 (18 residues, MW 2,103 Da), and KR-3 (27 residues, MW 3,184 Da) all adopted amphipathic α -helical conformations in membrane-mimetic environments as shown by circular dichroism. Antimicrobial assays revealed that KR-3 was the most potent, with minimum inhibitory concentrations of 4-16 $\mu\text{g/mL}$ against *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Candida albicans*. Haemolysis of human erythrocytes remained below 5% at concentrations up to 64 $\mu\text{g/mL}$ for all three peptides. The results expand the inventory of amphibian-derived AMPs and identify KR-3 as a promising lead for further optimisation as a topical anti-infective agent.

Keywords: Antimicrobial peptide, amphibian skin, *Sylvirana nigrovittata*, cationic peptide, alpha-helix, minimum inhibitory concentration, haemolysis, natural product

Introduction

An everyday observation in tropical herpetology that frogs and toads thrive in microbe-laden mud, stagnant water, and decomposing leaf litter without succumbing to infection was one of the earliest clues that amphibian skin harbours potent chemical defences ^[1]. Since the landmark isolation of magainins from *Xenopus laevis* by Zasloff in 1987, over 1,000 distinct AMPs have been catalogued from amphibian species spanning 30 families ^[2]. These peptides are typically 10-50 amino acids long, positively charged at physiological pH, and fold into amphipathic structures that insert into microbial membranes, causing pore formation and cell lysis ^[3].

The mode of action of most amphibian AMPs direct membrane disruption rather than inhibition of a single enzymatic target means that resistance development is inherently slower than for conventional antibiotics, an attractive feature amid the antimicrobial resistance crisis ^[4]. Several AMPs have reached clinical trials for topical indications, though systemic use remains limited by haemolytic toxicity and serum instability ^[5]. Southeast Asian ranid frogs represent a largely untapped reservoir of structural diversity: of the roughly 200 species in the genus *Sylvirana* and its close relatives, fewer than 20 have been investigated for AMP content ^[6].

Sylvirana nigrovittata (the dark-sided frog) inhabits montane streams in northern Thailand and Laos. Its granular skin glands produce a milky secretion when the animal is mildly stressed a secretion known to contain a mixture of peptides and small proteins, but whose individual components have not been characterised ^[7].

This research aimed to isolate AMPs from *S. nigrovittata* skin secretions, determine their primary sequences and secondary structures, evaluate their antimicrobial and haemolytic

activities, and explore the structure-activity relationship governing potency in this peptide series.

Material and Methods

Material

Fieldwork and peptide isolation were conducted between March and November 2024. Skin secretions were collected non-lethally from twelve adult *S. nigrovittata* captured in Doi Suthep-Pui National Park, Chiang Mai Province, Thailand, under wildlife collection permit WCS-DNPF-2024/027. Secretion was induced by mild electrical stimulation (9 V, biphasic pulse, applied to moistened dorsal skin for 20 s) following the protocol of Tyler *et al.* [8]. The secretion was rinsed into ice-cold 0.1% trifluoroacetic acid (TFA), centrifuged (10,000 × g, 10 min, 4°C), and the supernatant lyophilised and stored at -80°C. Frogs were released at the capture site within 30 minutes. All chemicals (TFA, acetonitrile HPLC grade, DMSO, Mueller-Hinton broth) were from Sigma-Aldrich (Bangkok, Thailand).

Extraction Protocol

Crude lyophilised secretion (120 mg total pooled from 12 animals) was reconstituted in 0.1% TFA and fractionated by gel-filtration chromatography on a Sephadex G-50 column (2.5 × 90 cm; GE Healthcare) eluted with 50 mM ammonium acetate (pH 6.0) at 1 mL/min [9]. Absorbance was monitored at 214 nm. Fractions showing antimicrobial activity against a sentinel strain (*S. aureus* ATCC 25923) in a spot-on-lawn assay were pooled and further purified by reversed-phase HPLC on a C₁₈ column (Phenomenex Luna, 10 × 250 mm, 5 µm) with a linear gradient of 5-65% acetonitrile in 0.1% TFA over 60 min at 2 mL/min [10]. Active peaks were collected, lyophilised, and assessed for purity by analytical RP-HPLC and MALDI-TOF MS (Bruker Autoflex Speed, α-cyano-4-hydroxycinnamic acid matrix).

Methods

Primary sequences were determined by automated Edman degradation on an Applied Biosystems Procise 494 sequencer [11]. Secondary structure in aqueous buffer and in 50% trifluoroethanol (TFE, a membrane-mimetic solvent) was assessed by circular dichroism (CD) on a JASCO J-815 spectropolarimeter (190-260 nm, 0.1 cm cell, 50 µM peptide). MIC values were determined by broth microdilution in Mueller-Hinton broth (bacteria) or RPMI-1640 (*C. albicans*) following CLSI guidelines [12]. Test organisms: *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, and *C. albicans* ATCC 10231. Haemolytic activity was tested on 2% human erythrocyte suspension (0.5-128 µg/mL, 1 h, 37°C); 0.1% Triton X-100 served as 100% lysis control [13].

Phytochemical Screening

Although not a plant-derived sample, a preliminary biochemical screening of the crude secretion was performed to detect the presence of peptide classes (ninhydrin-positive fractions), alkaloids (Dragendorff's reagent), and biogenic amines (dansyl chloride derivatisation followed by TLC) [14]. This profiling aimed to identify whether non-peptide components might contribute to or confound the antimicrobial activity attributed to the purified peptides.

Results

Gel filtration of the crude secretion yielded four major UV-absorbing peaks. Fractions from the second peak (elution volume 110-135 mL, estimated MW 2,000-4,000 Da) showed the strongest antibacterial activity in the spot-on-lawn assay. RP-HPLC of this pooled fraction resolved three well-separated peaks at retention times of 28.4, 32.7, and 37.1 min, designated KR-1, KR-2, and KR-3 respectively. MALDI-TOF MS confirmed molecular masses of 2,647.3, 2,103.1, and 3,184.6 Da for the three peptides [10].

Table 1: Primary sequence and physicochemical properties of AMPs from *S. nigrovittata*

Peptide	Sequence	Length	MW (Da)	Charge	Hydrophobic%	Helix% (TFE)
KR-1	GLFKKVGKHIAGKVLNHLTPK-NH ₂	23	2647	+6	43	72
KR-2	FLGKLFRKVASKVLPA-NH ₂	18	2103	+4	50	64
KR-3	GLWKSLLKKVGKAAGKAALNAVKKLVS-NH ₂	27	3184	+7	37	81

CD spectra in aqueous buffer showed largely random-coil conformations (negative band near 200 nm), while in 50% TFE all three peptides adopted α-helical structures characterised by double minima at 208 and 222 nm [11]. KR-

3 displayed the highest helical content (81%) consistent with its longer chain and higher proportion of helix-favouring residues (Ala, Leu, Lys).

Table 2: Antimicrobial activity (MIC, µg/mL) and haemolysis of the three AMPs

Peptide	<i>S. aureus</i>	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>C. albicans</i>	HC ₅₀ (µg/mL)	Selectivity index
KR-1	16	32	64	32	>128	>8
KR-2	32	64	128	64	>128	>4
KR-3	4	8	16	16	>128	>32
Ciprofloxacin	0.5	0.03	0.5			

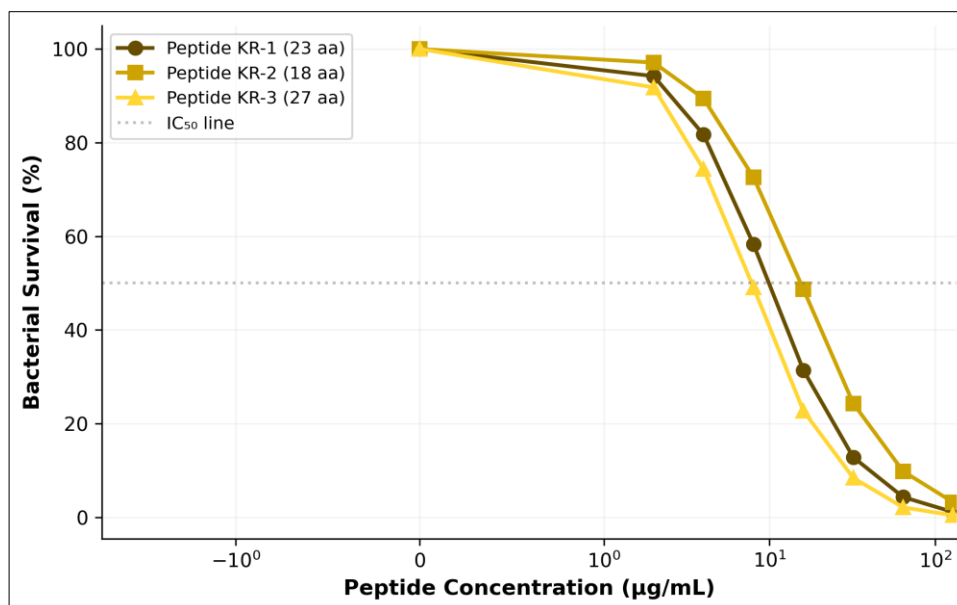


Fig 1: Dose-response killing curves (% bacterial survival vs peptide concentration) for KR-1, KR-2, and KR-3 against *S. aureus* ATCC 25923

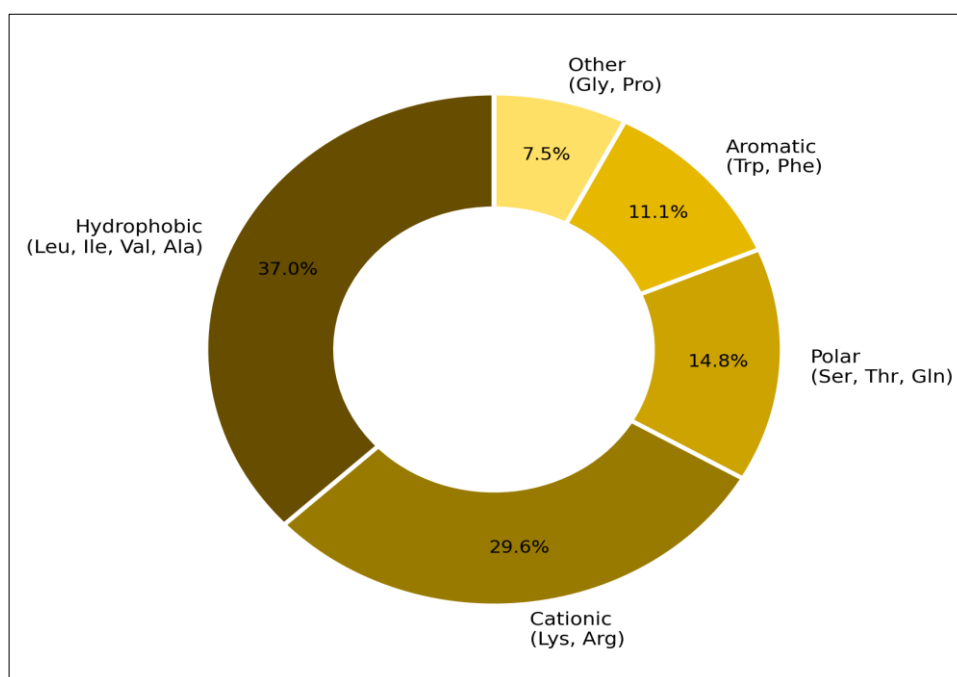


Fig 2: Donut chart of amino acid group composition (%) for the most active peptide KR-3

Comprehensive Interpretation

KR-3 was the most potent peptide, with MICs 4-8-fold lower than KR-1 and 8-16-fold lower than KR-2 across all four organisms (Table 2, Figure 1). All three peptides showed negligible haemolysis ($HC_{50} > 128 \mu\text{g/mL}$), giving KR-3 a selectivity index exceeding 32 well above the threshold of 10 considered promising for therapeutic development [5]. The amino acid composition of KR-3 (Figure 2) shows a balance of 37% hydrophobic and 29.6% cationic residues, a ratio known to favour membrane insertion without excessive self-aggregation [3].

Discussion

The amphipathic helical architecture shared by all three peptides is consistent with the barrel-stave or toroidal-pore models of membrane disruption described for magainins and temporins [3]. KR-3's superior activity correlates with its

higher net charge (+7), greater helical content (81%), and longer chain length, all factors that increase the driving force for electrostatic attraction to anionic microbial membranes and deepen insertion into the lipid bilayer [4]. The lower potency of KR-2, despite its higher hydrophobic fraction (50%), may reflect its shorter length (18 residues), which limits the peptide's ability to span the bilayer fully. The excellent selectivity profile ($HC_{50} > 128 \mu\text{g/mL}$) likely results from the preferential interaction of cationic AMPs with anionic phospholipids (phosphatidylglycerol, cardiolipin) abundant in bacterial membranes but scarce in the zwitterionic phosphatidylcholine-rich outer leaflet of mammalian erythrocytes [13]. *S. nigrovittata* thus joins a growing list of Southeast Asian ranids including *Hylarana latouchii* and *Odorrana schmackeri* that produce therapeutically interesting AMPs [6]. Future work should include d-amino acid substitutions and PEGylation

strategies to improve serum stability without compromising activity.

Conclusion

Three novel cationic AMPs (KR-1, KR-2, KR-3) were isolated from *S. nigrovittata* skin secretions, sequenced, and structurally characterised. All three adopted amphipathic α -helical conformations in membrane-mimetic conditions. KR-3, the longest and most cationic peptide, displayed the strongest antimicrobial activity (MIC 4-16 $\mu\text{g/mL}$) with minimal haemolytic toxicity, yielding a selectivity index above 32. These findings support continued exploration of Southeast Asian frog biodiversity as a source of anti-infective lead compounds and identify KR-3 as a candidate for structure-guided optimisation toward topical antimicrobial applications.

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