Peptides with differential cytolytic activity from skin secretions of the lemur leaf frog *Hylomantis lemur* (Hylidae: Phyllomedusinae) ☆

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Abstract

Two peptides with differential cytolytic activity against bacteria, a fungus pathogenic to amphibians, and mammalian cells were isolated from norepinephrine-stimulated skin secretions of the Lemur leaf frog *Hylomantis lemur* Boulenger, 1882. Dermaseptin-L1 (GLWSKIKEAAKAAGKAALNAVTGLVNQGDQPS) was active against the Gram-negative bacterium *Escherichia coli* (MIC $= 8 \, \mu M$) but inactive against the Gram-positive bacterium *Staphylococcus aureus*. This peptide inhibited growth of zoospores of the chytrid fungus *Batrachochytrium dendrobatidis* at concentrations above $25 \, \mu M$ but did not completely inhibit growth at $100 \, \mu M$. Phylloseptin-L1 (LLGMIPLAISAISALSKL.NH$_2$) was active against *S. aureus* (MIC $= 8 \, \mu M$) but was inactive against *E. coli*. This peptide also inhibited growth of *B. dendrobatidis* zoospores at concentrations above $25 \, \mu M$ with complete inhibition at $100 \, \mu M$. Dermaseptin-L1 showed selective cytolytic activity against HepG2 human hepatoma-derived cells (LC$_{50} = 45 \, \mu M$) compared with human erythrocytes (LC$_{50} = 200 \, \mu M$) whereas phylloseptin-L1 was approximately equipotent against both HepG2 cells (LC$_{50} = 35 \, \mu M$) and erythrocytes (LC$_{50} = 40 \, \mu M$).
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Keywords: Amphibian; Antimicrobial; Cytolysis; Hepatoma; Chytrid

1. Introduction

Cytolytic peptides are synthesized in granular glands present in the skins of a wide range of anuran species (frogs and toads) and constitute an important component of the animal's system of innate immunity (Hancock, 2001). The peptides serve to

protect the organism against invasion by pathogenic bacteria and fungi (Rollins-Smith and Conlon, 2005) and may also function to deter ingestion by predators. The peptides are released into skin secretions, often in very high concentrations, in response to infection or stress (Davidson et al., 2007). In the laboratory or in the field, stimulation may be achieved under conditions that do not cause distress by injection of norepinephrine (Nutkins and Williams, 1989) or by mild transcutaneous electro-stimulation (Tyler et al., 1992).

The Phyllomedusinae sub-family of the Hylidae family comprises 56 species and has proved to be a rich source of peptides with cytolytic activity. These peptides may be grouped together on the basis of limited structural similarity and the following families have been identified: (a) dermaseptins, (b) dermatoxins, (c) phylloxins, (d) phylloseptins, and (e) Gly-Leu-rich peptides (reviewed in Nicolas and Amiche, 2006). The members of the different families differ appreciably in both primary structures and biological activities, but strong conservation of the amino acid sequences of the signal peptide and N-terminal pro-regions of the biosynthetic precursors of the peptides demonstrates that they are related evolutionarily (Vanhoye et al., 2003).

The Lemur leaf frog *Hylomantis lemur* Boulenger, 1882 (formerly known as *Phyllomedusa lemur*) (Favovich et al., 2005) is a small (length 30–45 mm) anuran that inhabits humid, mid-elevation regions (roughly 500–1500 m) in Panama, Costa Rica, and Colombia. The animals are largely nocturnal and prefer a habitat of less-dense vegetation, where they hide on the underside of leaves a few meters above ground. Although once considered to be relatively common, populations have declined drastically during the past 20 years and the species is now listed as endangered. The present study describes the isolation from norepinephrine-stimulated skin secretions of *H. lemur* of two peptides with varying degrees of cytolytic activity against bacteria, fungi, and mammalian cells that were identified as belonging to the dermaseptin and phylloseptin families.

2. Experimental

2.1. Collection of skin secretions

The collection of norepinehrine-stimulated skin secretions from adult specimens of *H. lemur* (n = 9)

in Omar Torrijos National Park, Coclé, Panama and partial purification on Sep-Pak C-18 cartridges has been described in detail previously (Woodhams et al., 2006). After collection of secretions, all animals were released at the exact site of collection. Permits for collection of samples were provided through the Smithsonian Tropical Research Institute from the Autoridad Nacional del Ambiente, Republic of Panama.

2.2. Antibacterial assays

Purification of the peptides was monitored by incubating lyophilized aliquots of chromatographic effluent in Mueller–Hinton broth (50 µl) with an inoculum (50 µl of 10^6 colony forming units ml^{-1}) from a log-phase culture of reference strains *Staphylococcus aureus* (ATCC 25923) and *Escherichia. coli* (ATCC 25726) in 96-well microtiter cell-culture plates for 18 h at 37 °C in a humidified atmosphere of air. After incubation, the absorbance at 630 nm of each well was determined using a microtiter plate reader. In order to monitor the validity and reproducibility of the assays, incubations were carried out in parallel with increasing concentrations of ampicillin. Minimum inhibitory concentration (MIC) was measured by a standard microdilution method (National Committee for Clinical Laboratory Standards, 1997) and was taken as the lowest concentration of peptide where no visible growth was observed.

2.3. Antifungal assays

Batrachochytrium dendrobatidis isolate 197 from the blue poison dart frog, *Dendrobates azureus* (Longcore et al., 1999) was maintained in culture and zoospores were plated with or without peptides as described previously (Rollins-Smith et al., 2002a, b). Briefly, 5×10^5 zoospores in a volume of 50 µl broth were plated in replicates of five in a 96-well flat bottom microtitre plate (Costar 3596, Corning Inc., Corning NY, USA) with or without addition of 50 µl serial dilutions of peptides in sterile HPLC-grade water. To determine maximal growth (positive control for growth), some wells received 50 µl of HPLC-grade water without peptide. To determine the value for maximal inhibition (negative control for growth) some cultures were treated by temperature shock (60 °C for 10 min) to induce cell death. Growth at 1–7 days (23 °C) was measured as increased optical density at 492 nm
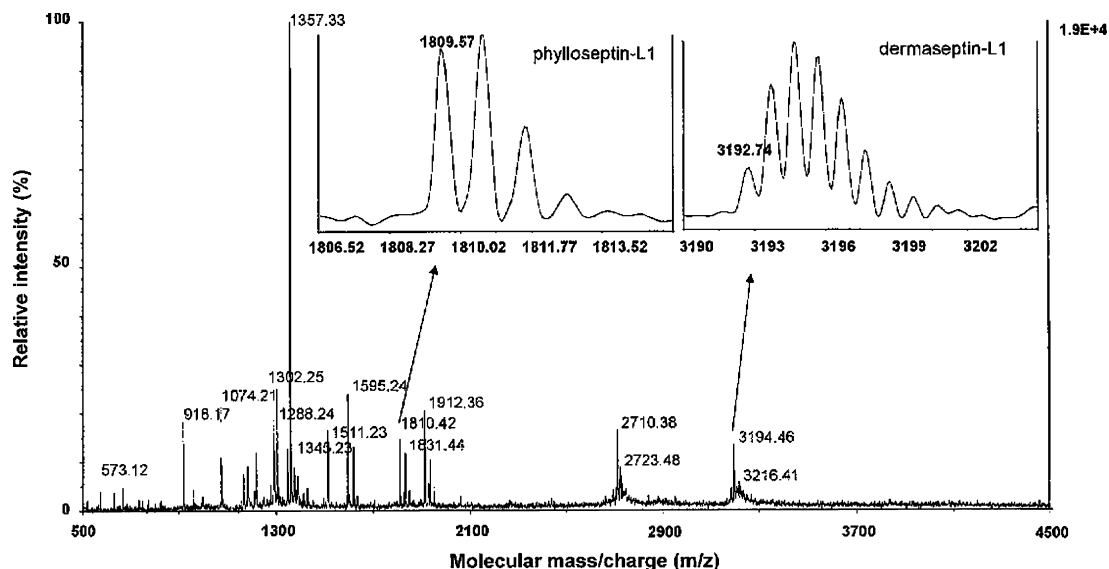
Fig. 1. MALDI-TOF mass spectrum of skin peptides recovered from *Hylomantis lemur* after administration of norepinephrine. Insets represent mass signals and isotopic peaks for phylloseptin-L1 (*m/z* 1809.57) and dermaseptin-L1 (*m/z* 3192.74). Note that several peptides with high relative abundance have not yet been identified.

(OD492). MIC is defined as the lowest concentration at which no growth was detectable.

2.4. Cytolytic assays

Peptides in the concentration range 10–320 μM, or lyophilized fractions of chromatographic effluent (50 μl) were incubated in duplicate with washed human erythrocytes (2×10^7 cells) from a healthy donor in Dulbecco's phosphate-buffered saline, pH 7.4 (100 μl) for 1 h at 37 °C. After centrifugation (12,000g for 15 s), the absorbance at 450 nm of the supernatant was measured. A parallel incubation in the presence of 1% v/v Tween-20 was carried out to determine the absorbance associated with 100% hemolysis. The LC_{50} value was taken as the mean concentration of peptide producing 50% hemolysis in three independent experiments.

HepG2 human hepatoma-derived cells were purchased from the American Type Culture Collection (Manassas VA, USA) and cultured as previously described (Bai and Cederbaum, 2006). Cells (2×10^7) were seeded into 96 well microtiter plates and grown to 90% confluence in minimum essential medium containing 10% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine in an humidified atmosphere of 5% CO_2 at 37 °C. One hour before incubation, the medium in each well was replaced with minimum essential medium containing 5% fetal calf serum.

Peptides in the concentration range 10–320 μM were incubated with the cells for 1 h at 37 °C in duplicate. Cytolytic activity was determined by measurement of lactate dehydrogenase using a CytoTox96 nonradioactive cytotoxicity assay kit (Promega, Madison, WI, USA) according to the manufacturer's protocol. The LC_{50} value was taken as the mean concentration of peptide producing 50% cytolysis in three independent experiments.

2.5. MALDI–TOF MS analysis of the natural mixture of skin peptides

The mixture of skin peptides was analyzed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI–TOF MS). Secretions, after partial purification on Sep-Pak cartridges, were spotted onto the sample plate at 1 mg/ml before adding an equal volume of α-cyano-4-hydroxycinnamic acid matrix solution (Sigma-Aldrich, St. Louis, MO). An Applied Biosystems Elite spectrometer was operated in reflector, delayed extraction and positive ion mode. For external calibration, a series of peptide standards (Sigma-Aldrich) were applied. Mass spectra were acquired across the range of *m/z* (mass to charge ratio) 500–10,000. Dermaseptin-L1 and phylloseptin-L1 were identified as distinctive peaks within the skin peptide profile.

2.6. Peptide purification

The lyophilized skin secretions (approximately 83 mg), after partial purification on Sep-Pak cartridges, were redissolved in 0.1% (v/v) trifluoroacetic acid/water (2 ml) and injected onto a (1.0 × 25 cm) Vydac 218TP510 (C-18) reversed-phase HPLC column (Separations Group, Hesperia, CA, USA) equilibrated with 0.1% (v/v) trifluoroacetic acid/water at a flow rate of 2.0 ml/min. The concentration of acetonitrile in the eluting solvent was raised to 21% (v/v) over 10 min and to 63% (v/v) over 60 min using linear gradients. Absorbance was monitored at 214 and 280 nm and fractions (1 min) were collected. The abilities of freeze-dried aliquots (50 µl) of the fractions to inhibit the growth of *S. aureus* and *E. coli* and to lyse human erythrocytes were determined as described in the previous sections.

Aliquots of fractions from the major UV-absorbing peaks with cytolytic activity against bacteria and/or human erythrocytes (peaks 1 and 2 in Fig. 1) were rechromatographed on a (1.0 × 25 cm) Vydac 214TP54 (C-4) column. The concentration of acetonitrile in the eluting solvent was raised from 21% to 56% over 50 min and the flow rate was 2.0 ml/min. The peptides were purified to near homogeneity by a final chromatography on a 0.46 × 25 cm Vydac 219TP54 phenyl column. The concentration of acetonitrile in the eluting solvent was raised from 28% to 56% over 50 min and the flow rate was 1.5 ml/min.

2.7. Structural characterization

The primary structures of the peptides were determined by automated Edman degradation using a model 494 Procise sequenator (Applied Biosystems, Foster City, CA, USA). Amino acid composition analyses were performed by the University of Nebraska Medical Center Protein Structure Core Facility (Omaha, NE, USA). MALDI–TOF MS was carried out using a Voyager DE-PRO instrument (Applied Biosystems) that was operated in reflector mode with delayed extraction and the accelerating voltage in the ion source was 20 kV. The instrument was calibrated with peptides of known molecular mass in the 2000–4000 Da range. The accuracy of mass determinations was $\pm 0.02\%$.

2.8. Peptide synthesis

Dermaseptin-L1 and phylloseptin-L1 were supplied in crude form by GL Biochem Ltd. (Shanghai,

China) and were purified to near homogeneity by reversed-phase HPLC on a (2.2 × 25 cm) Vydac 218TP1022 (C-18) column equilibrated with acetonitrile/water/trifluoroacetic acid (21.0/78.9/0.1, v/v/v) at a flow rate of 6 ml/min. The concentration of acetonitrile was raised to 56% (v/v) over 60 min using a linear gradient. Absorbance was measured at 214 and 280 nm and the major peak in the chromatogram was collected manually. For both peptides, the monoisotopic molecular masses determined by electrospray mass spectrometry were consistent with the masses calculated from the proposed structures. The purity of the peptides was >98%.

3. Results

3.1. MALDI–TOF MS analysis of the natural mixture of skin peptides

MALDI–TOF MS analysis of skin peptide mixtures provides a rapid method to assess the relative complexity of a skin peptide mixture and an approximate estimate of the relative abundance of each peptide. Analysis of the skin peptides eluted from C-18 Sep-Pak cartridges revealed a complex pattern of peptides with masses between 500 and 3500 (Fig. 1). The antimicrobial peptides that were later identified as phylloseptin-L1 and dermaseptin-L1 are indicated by arrows.

3.2. Purification of the peptides

The skin secretions, after concentration and partial purification on Sep-Pak cartridges, were chromatographed on a Vydac C-18 semipreparative reverse-phase HPLC column (Fig. 2). The prominent peak designated 1 (subsequently shown to contain dermaseptin-L1) showed growth-inhibitory activity against *E. coli* and *B. dendrobatidis* (Fig. 4) but was inactive against *S. aureus* and only weakly hemolytic. Peak 2 (subsequently shown to contain phylloseptin-L1) showed growth-inhibitory activity against *S. aureus* and *B. dendrobatidis* (Fig. 4), was strongly hemolytic, but was inactive against *E. coli* (Figs. 3 and 4).

Purification of the active component in peak 1 (dermaseptin-L1) and peak 2 (phylloseptin-L1) on a semipreparative Vydac C-4 column is shown in Fig. 3. The peptides were purified to near homogeneity, as assessed by a symmetrical peak shape and mass spectrometry, by a final chromatography
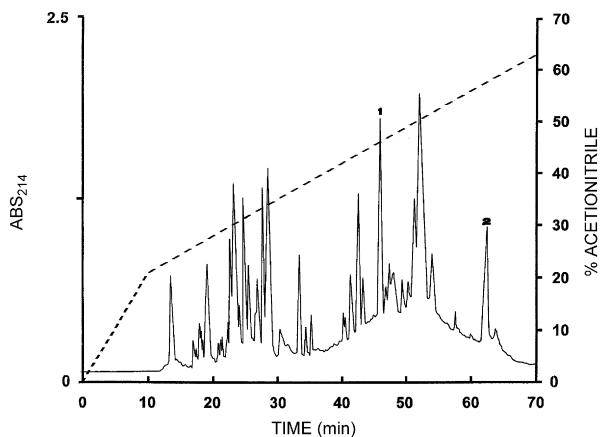
Fig. 2. Reversed-phase HPLC on a semipreparative Vydac C-18 column of skin secretions from *H. lemur* after partial purification on Sep-Pak cartridges. Peptides in peak 1 (dermaseptin-L1) and peak 2 (phylloseptin-L1) showed cytolytic activity and were subjected to further purification. The dashed line shows the concentration of acetonitrile in the eluting solvent.

on an analytical Vydac phenyl column. The final yields of purified peptides were: dermaseptin-L1, 14 nmol and phylloseptin-L1, 25 nmol.

3.3. Structural characterization

The primary structures of the peptides were established by automated Edman degradation as dermaseptin-L1: GLWSKIKEAAKAAGKAA-LNAVTGLVNQGDQPS, and phylloseptin-L1: LLGMIPLAISAISALSKL. The observed molecular masses of the peptides, determined by MALDI–TOF mass spectrometry, are consistent with the proposed sequences and demonstrate that phylloseptin-L1 is C-terminally α-amidated (dermaseptin-L1: observed molecular mass 3192.8 a.m.u., calculated molecular mass 3192.7 a.m.u.; phylloseptin-L1: observed molecular mass 1809.0 a.m.u., calculated molecular mass 1809.1 a.m.u for the C-terminally α-amidated form of the peptide).

It was not possible to determine the C-terminal amino acid residue in phylloseptin-L1 by Edman degradation but amino acid composition analysis demonstrated that this residue was leucine (Found: Ser 3.0 (3), Pro 1.0 (1), Gly 1.1 (1), Ala 3.1 (3), Met 1.0 (1), Ile 2.7 (3), Leu 4.7 (5), Lys 1.1 (1) residues/mol peptide). The figures in parentheses show the number of residues predicted from the proposed sequence. The amino acid composition of dermaseptin-L1 was consistent with its proposed structure.
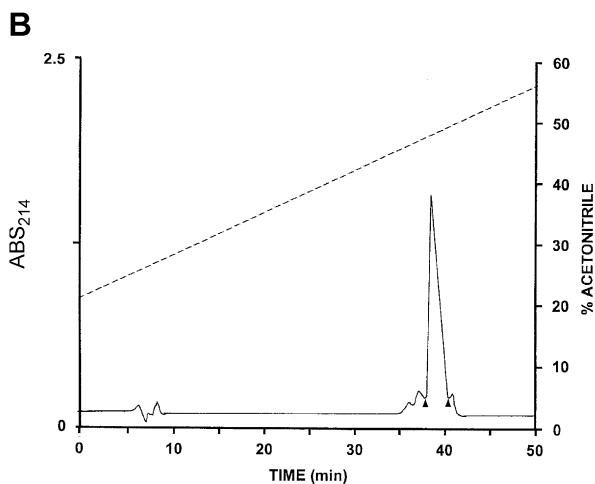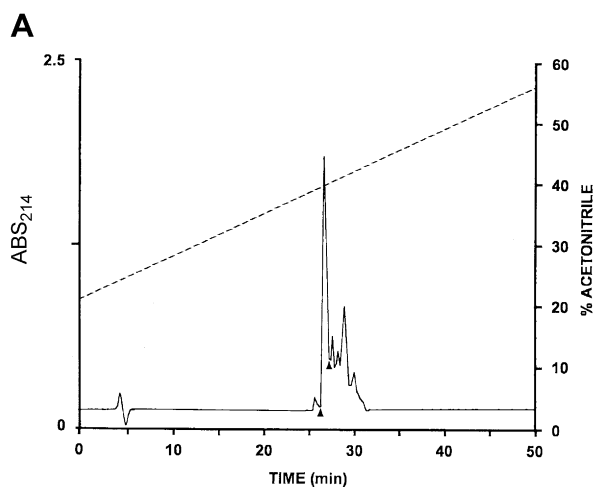

Fig. 3. Purification on a Vydac C-4 column of (A) dermaseptin-L1 and (B) phylloseptin-L1. The arrowheads show where peak collection began and ended.

3.4. Cytolytic activity

The MIC of dermaseptin-L1 and phylloseptin-L1 against *E. coli*, *S. aureus*, and *B. dendrobatidis* and their cytolytic activities (LC_{50} values) against human erythrocytes and HepG2 human hepatoma-derived cells are shown in Table 1.

4. Discussion

This study has identified two peptides with differential cytolytic activity against bacteria, fungi, and mammalian cells in skin secretion of the Panamanian frog, *H. lemur*. Structural characterization of the peptides has identified them as members of the dermaseptin and phylloseptin families. The dermaseptins are an extensive family

of peptides whose members have been isolated from the skins of a range of species belonging to the Phyllomedusinae [*Phyllomedusa sauvagei* (Mor and Nicolas, 1994a; Chen et al., 2003), *Phyllomedusa bicolor* (Charpentier et al., 1998), *Phyllomedusa oreades* (Brand et al., 2002), *Phyllomedusa distincta* (Brand et al., 2002), *Phyllomedusa hypochondrialis* (Brand et al., 2006) *Pachymedusa dacnicolor* (Wechselberger, 1998), *Agalychnis annae* (Wechselberger, 1998), and *Agalychnis callidryas* (Vanhoye et al., 2003)]. The primary structure of dermaseptin-L1 from *H. lemur* is compared with the structures of the
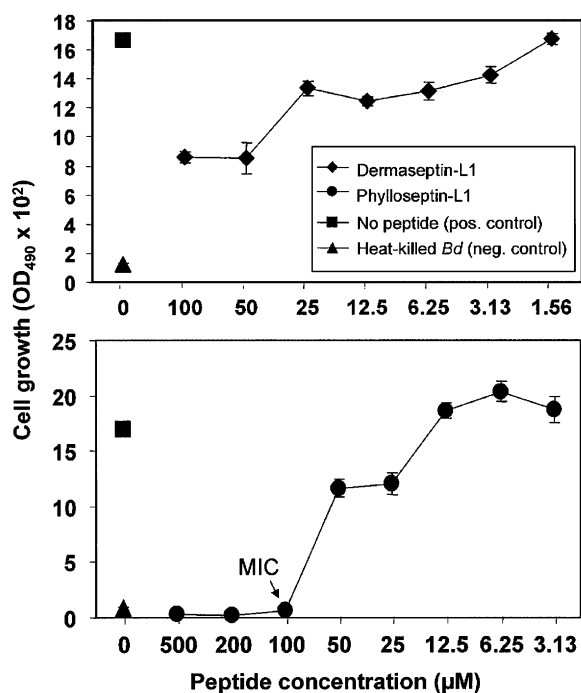

Fig. 4. Growth inhibition of *B. dendrobatidis* zoospores (type isolate 197) by synthetic dermaseptin-L1 (top panel) and phylloseptin-L1 (bottom panel). Growth was measured at seven days of culture as optical density at 490 nm (OD_{490}). Each data point represents the mean ± standard error (SE) of five replicate wells. If no error bar is shown, the SE was less than the diameter of the symbol. Minimal inhibitory concentration (MIC) is the lowest concentration of peptide at which no significant fungal cell growth was detected.

eight members of the dermaseptin family identified in *P. sauvagei* in Fig. 5. The dermaseptins vary appreciably both in length and amino acid sequence but have the propensity to form an amphipathic α-helical conformation in a membrane-mimetic solvent. The peptides characterized to-date contain a Trp residue at position 3 and a conserved motif (Ala-Xaa-Lys-Ala-Ala-Xaa-Xaa-Ala) in the central region of the molecule. More recently, a peptide termed dermaseptin-S9 (GLRSKIWLWVLLMIW-QESNKFKKM) has been identified in *P. sauvagei* skin secretions but this component lacks these conserved structural features (Lequin et al., 2006).

Dermaseptins-S1, -S3, and -S5 show broad-spectrum antimicrobial activity with relatively high potency against a range of Gram-positive and Gram-negative bacteria but are only weakly hemolytic ($LC_{50} > 100\,\mu M$) whereas dermaseptin-S4 also shows broad-spectrum antibacterial activity but is very strongly hemolytic ($LC_{50} < 1\,\mu M$) (Nicolas and Amiche, 2006). As shown in Table 1, dermaseptin-L1 is active against the Gram-negative bacteria *E. coli* and *B. dendrobatidis* but inactive against the Gram-positive bacteria *S. aureus*. It is proposed that the presence of a lysine residue at position 5 results in a decrease in amphipathicity that is responsible for the loss of activity against *S. aureus*. It has been postulated that a stabilized amphipathic α-helical conformation is an absolute requirement for cidal activity against Gram-positive bacteria whereas the structural requirements for activity against Gram-negative bacteria are less stringent. Provided that cationicity and hydrophobicity are maintained, peptides with impaired amphipathicity may be still active (Giangaspero et al., 2001). Studies with truncated analogs of dermaseptin-S3 have shown that the (1–16) fragment retains full antimicrobial potency (Mor and Nicolas, 1994b). A Schiffer–Edmundson wheel representation (Schiffer and Edmundson, 1967) of the N-terminal domains of dermaseptin-L1 (residues 1–16) and dermaseptin-S3 (residues 1–16) (Fig. 6) shows that the amphipathicity of the helix in dermaseptin-L1 is reduced.

Table 1

Minimum inhibitory concentration (μM) against bacteria and the chytrid fungus *B. dendrobatidis* and LC_{50} values (μM) against human erythrocytes and hepatoma-derived HepG2 cells of synthetic replicates of dermaseptin-L1 and phylloseptin-L1

	E. coli	*S. aureus*	*B. dendrobatidis*	Erythrocytes	HepG2 cells
Dermaseptin-L1	8	>128	>100	200	45
Phylloseptin-L1	>128	8	100	40	35

A

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Dermaseptin L1      GLWSKI    KEAAKAAGKAALNAVTGLVNQGDQPS
Dermaseptin S1      ALWKTMLKKLGTMALHAGKAALGAAADTISQGTQ
Dermaseptin S2      ALWFTMLKKLGTMALHAGKAALGAAANTISQGTQ
Dermaseptin S3      ALWKNMLK   GIGKLAGKAALGAVKKLVGAES
Dermaseptin S4      ALWMTLLK   KVLKAAAKAALNAVLVGANA
Dermaseptin S5      GLWS IKTAGKSVAKAAAKAAVKAVTNAV
Dermaseptin S6      GLWSKIKTA KEAAKAAAKAAGKAALNAVSEAI.NH₂
Dermaseptin S7      GLWKS   LLKNVGEAAGKAALNAVTDMVNQ.NH₂
Dermaseptin S8      ALWKTMLKKLGTVALHAGKAALGAAADTISQ.NH₂
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B

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Phylloseptin L1     LLGMIPLAISAISALSKL.NH₂
Phylloseptin 1      FLSLIPHAINAVSAIAKHN.NH₂
Phylloseptin 2      FLSLIPHAINAVSTLVHHF.NH₂
Phylloseptin 3      FLSLIPHAINAVSALANHG.NH₂
Phylloseptin 4      FLSLIPHAINAVSTLVHHSG.NH₂
Phylloseptin 5      FLSLIPHAINAVSAIAKHS.NH₂
Phylloseptin 6        SLIPHAINAVSAIAKHF.NH₂
Phylloseptin 7      FLSLIPHAINAVSAIAKHF.NH₂
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Fig. 5. A comparison of the primary structures of (A) dermaseptins from *H. lemur* (L-1) and *P. sauvagei* (S1–S8), and (B) phylloseptins from *H. lemur* (L-1), *P. hypochondrialis* (-1-, -2, -3, -6, -7) and *P. oreades* (-4, -5). Conserved residues are shaded and gaps have been introduced in some sequences to maximize structural similarity.

In this peptide, the Lys^5 residues segregates on the opposite face to Lys^7, Lys^{11}, and Lys^{15} whereas in dermaseptin-S3, the Lys^4, Lys^8, Lys^{12}, and Lys^{16} residues are located on the same face of the helix.

The phylloseptins are a family of structurally related peptides identified in the skin secretions of the Brazilian tree frogs, *P. hypochondrialis* (Leite et al., 2005; Conceicao et al., 2006) and *P. oreades* (Leite et al., 2005). Their primary structures are compared with that of phylloseptin-L1 in Fig. 5. In contrast to dermaseptin-L1, phylloseptin-L1 is active against the Gram-positive bacteria *S. aureus* and *B. dendrobatidis* but not *E. coli*. It has been suggested that the secretion of multiple peptides with different specificities against bacteria serves to protect phyllomedusid frogs against invasion by a range of pathogens (Mor et al., 1994). Consistent with this idea, cosecretion of dermaseptin-L1 and phylloseptin-L1 by *P. lemur* will provide more comprehensive protection against bacteria and *B. dendrobatidis* than either peptide alone. Populations of *H. lemur* have declined in Costa Rica (Pounds et al., 1997) and Western Panama (Lips, 1999). At Omar Torrijos National Park, we predicted this species would survive the emergence of chytridiomycosis (Woodhams et al., 2006). However, although the population has declined (Lips et al., 2006), some survivors have since been observed at this location (Karen Lips, personal communication). It is clear that the emergence of chytridiomycosis causes declines in this species despite the ability to secrete significant quantities of antimicrobial skin peptides. The activity of these peptides against *B. dendrobatidis* demonstrated *in vitro* (Fig. 4 and Table 1) may not represent a sufficient *in vivo* defense.

Several naturally occurring frog skin peptides have been shown to possess cytolytic activity against tumor cells. Examples include the magainins from *Xenopus laevis* skin that are active against a range of human lung cancer (Ohsaki et al., 1992) and other tumor (Cruciani et al., 1991) cell lines, and gaegurin-6 from the skin of *Rana rugosa* that is active against multi-drug resistant human breast cancer cell lines (Kim et al., 2003). An earlier study in the investigator's laboratory identified a melittin-related peptide in the skin of *Rana tagoi* that was strongly cytolytic towards mouse
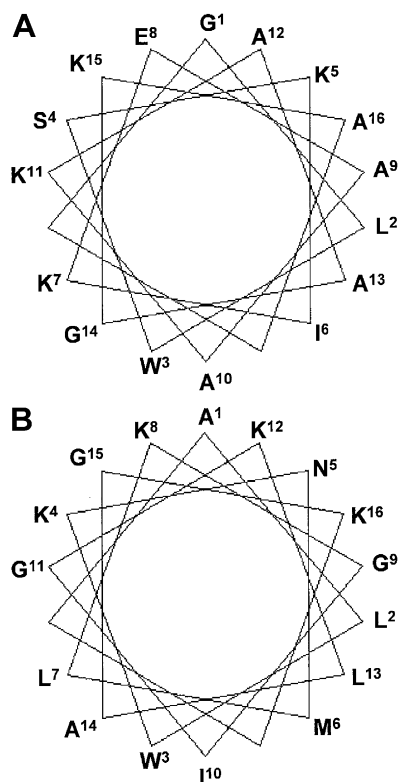
A

B

Fig. 6. A Schiffer–Edmundson helical wheel representation of (A) residues 1–16 of dermaseptin-L1 and (B) residues 1–16 of dermaseptin-S3.

EL4 T-lymphoma-derived cells ($LC_{50} = 14\,\mu M$) and human HeLa cervical cancer-derived cells ($LC_{50} = 8\,\mu M$) (Conlon et al., 2003). However, this particular peptide showed no selectivity toward tumor cells compared with non-neoplastic cells (LC_{50} against human erythrocytes $= 8\,\mu M$). The fivefold selectivity of dermaseptin-L1 for HepG2 neoplastic cells compared with erythrocytes (Table 1) suggests that the peptide may form the basis for the design of analogs with therapeutic potential as anti-cancer agents. Studies with model peptides composed of D- and L- leucines, lysines and arginines have demonstrated that it is possible to design analogs that display up to 16-fold higher toxicities against D122 lung carcinoma cells and B16 melanoma-derived cells than against non-neoplastic NIH-3T3 cells (Papo et al., 2003).

Most of the skin peptides of this endangered Panamanian frog remain to be characterized and their functions remain unknown. We describe two novel cytolytic peptides found in the skin secretions of this species. The potential anti-cancer and anti-

bacterial functions of the peptides demonstrate the importance of this species and biodiversity in general to human medicine and the urgency of the need for conservation of rapidly disappearing amphibians.

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