Available online at www.sciencedirect.com

SCIENCE DIRECT

Regulatory Peptides 129 (2005) 103–108

REGULATORY PEPTIDES

www.elsevier.com/locate/regpep

Dermatoxin and phylloxin from the waxy monkey frog, *Phyllomedusa sauvagei*: Cloning of precursor cDNAs and structural characterization from lyophilized skin secretion

Tianbao Chen, Brian Walker, Mei Zhou, Chris Shaw*

School of Pharmacy, Queen's University, 97 Lisburn Road, Belfast BT9 7BL Northern Ireland, UK

Received 30 November 2004; received in revised form 4 January 2005; accepted 27 January 2005
Available online 2 March 2005

Abstract

Amphibian skin is a morphologically, biochemically and physiologically complex organ that performs the wide range of functions necessary for amphibian survival. Here we describe the primary structures of representatives of two novel classes of amphibian skin antimicrobials, dermatoxin and phylloxin, from the skin secretion of *Phyllomedusa sauvagei*, deduced from their respective precursor encoding cDNAs cloned from a lyophilized skin secretion library. A degenerate primer, designed to a highly conserved domain in the 5′-untranslated region of analogous peptide precursor cDNAs from *Phyllomedusa bicolor*, was employed in a 3′-RACE reaction. Peptides with molecular masses coincident with precursor-deduced mature toxin peptides were identified in LC/MS fractions of skin secretion and primary structures were confirmed by MS/MS fragmentation. This integrated experimental approach can thus rapidly expedite the primary structural characterization of amphibian skin peptides in a manner that circumvents specimen sacrifice whilst preserving robustness of scientific data.

Keywords: Phyllomedusinae; Venom; Peptide; Mass spectroscopy; cDNA

1. Introduction

The emergence of multiple drug-resistant strains of many pathogenic microorganisms in recent years has stimulated the search for new classes of antimicrobial compounds that may have clinical applications [1–3]. Among these potential new leads are a group that have received increasing attention— the broad-spectrum antimicrobial polypeptides that are synthesized and stored in amphibian skin granular glands [4]. The presence of such peptides in amphibian skin secretions represents an important feature of the defense strategy of these vertebrates against micro-predator attack and some authors believe that they constitute the front-line barrier of a primitive cell-free immune system [5–7]. Stress or injury will result in secretion of the granular gland contents onto the skin surface of the amphibian—an effect mediated by sympathetic nervous system-induced contraction of surrounding myoepithelial cells [4,7]. The antimicrobial peptides frequently occur as multiple structurally related isoforms with each isoform potentially possessing differential activity against a range of pathogenic microorganisms [8]. Amphibian skin peptides have been the subject of intense research interest for many years from both academic and pharmaceutical groups due to their potential applications in biophysical research, biochemical taxonomy and in lead compound development for new pharmaceuticals [9,10].

The subfamily Phyllomedusinae contains three well-known genera, *Phyllomedusa*, *Agalychnis* and *Pachymedusa*, with the former containing the vast majority of known species [11]. Analysis of the skin secretions and/or skin extracts from different species of phyllomedusine frog

* Corresponding author. Tel.: +44 2890 972129; fax: +44 2890 247794.
E-mail address: chris.shaw@qub.ac.uk (C. Shaw).

0167-0115/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.regpep.2005.01.017

has led to the identification and characterization of a large number of peptides, leading Vittorio Erspamer to describe them as "huge storehouses of bioactive peptides" [12]. Indeed, a prototypical antimicrobial peptide family, the dermaseptins, originated from this amphibian genus [13]. The amino acid sequences of dermaseptins are highly variable but all are cationic due to a preponderance of lysine residues and most have the potential to form amphipathic helices either in aqueous solution or upon interaction with the lipid bilayer of bacterial cell membranes [1]. However, while the primary structures of dermaseptins are variable, the signal peptide and acidic spacer peptide domains of their precursors are highly conserved. In fact, this observation is extended to nucleotide sequences of cDNAs within 3′-and 5′-non-coding regions. Dermatoxin and phylloxin are two prototype narrow-spectrum antimicrobial peptides identified in *Phyllomedusa bicolor* skin [14,15]. Despite significant differences in primary structure with each other and with dermaseptins, the organization, nucleotide sequence of cloned cDNAs and primary structures of signal peptides and acidic spacer domains of respective precursors are remarkably highly conserved [16].

Here, we report the primary structures of dermatoxin and phylloxin homologs from the waxy monkey frog, *Phyllomedusa sauvagei*, as determined by "shotgun" cloning of respective precursor cDNAs from a library constructed from lyophilized skin secretion [17,18]. Subsequent deduction of the molecular masses of each mature processed peptide was used to locate respective molecules in LC/MS fractions and to confirm their predicted primary structures by either automated Edman degradation or MS/MS fragmentation. The peptides were named in accordance with previously established nomenclature as DRT-S and PLX-S, respectively.

2. Materials and methods

2.1. Specimen biodata and secretion acquisition

Phyllomedusa sauvagei (n=3) were obtained from a commercial source and had been captive bred in the United States. The frogs were adults on receipt (2 males 5 cm snout-to-vent length, 1 female 7 cm snout-to-vent length) and were settled into their new surroundings for 4 months prior to secretion harvesting. They were maintained in our purpose-designed amphibian facility at 20–25 °C under a 12 h/12 h light/dark cycle and fed multivitamin-loaded crickets three times per week. Skin secretion was obtained from the paratoid and tibial glands by gentle squeezing as mild transdermal electrical stimulation was found to be ineffective. The copious viscous white secretion was washed from the skin using deionized water, snap-frozen in liquid nitrogen and lyophilized. Lyophilizate was stored at −20 °C prior to analysis.

2.2. "*Shotgun*" *cloning of P. sauvagei dermatoxin and phylloxin cDNAs*

Five milligrams of lyophilized venom was dissolved in 1 ml of cell lysis/mRNA stabilization solution (Dynal, UK). Polyadenylated mRNA was isolated using magnetic oligo-dT beads as described by the manufacturer (Dynal Biotech, UK) and reverse-transcribed. The cDNA was subjected to 3′-RACE procedures to obtain full-length preprodermatoxin/preprophylloxin nucleic acid sequence data using a SMART-RACE kit (Clontech UK) essentially as described by the manufacturer. Briefly, the 3′-RACE reactions employed a NUP primer (supplied with the kit) and a sense primer (S1; 5′-TCTGAATTRYAAGMSCAR-ACATG-3′) that was designed to a highly conserved domain of the 5′-untranslated region of previously characterized homologous peptide cDNAs from *Phyllomedusa bicolor* [14,15]. The PCR cycling procedure was as follows. Initial denaturation step 60 s at 94 °C; 35 cycles, denaturation 30 s at 94 °C, primer annealing for 30 at 54 °C; extension for 180 s at 72 °C. PCR products were gel-purified and cloned using a pGEM-T vector system (Promega) and sequenced using an ABI 3100 automated sequencer.

2.3. Identification and structural analyses of deduced dermatoxin and phylloxin homologs

Five milligrams of lyophilized venom were dissolved in 0.5 ml of 0.05/99.5 (v/v) trifluoroacetic acid (TFA)/water and clarified of microparticulates by centrifugation. The supernatant was then subjected to LC/MS using a gradient formed from 0.05/99.5 (v/v) TFA/water to 0.05/19.95/80.0 (v/v/v) TFA/water/acetonitrile in 240 min at a flow rate of 1 ml/min. A Thermoquest gradient reversed phase HPLC system, fitted with an analytical column (Jupiter C-5, 5 μ particle, 300 Å pore, 250×10 mm, Phenomenex, UK) and interfaced with a Thermoquest LCQ™ electrospray ion-trap mass spectrometer, was employed. The effluent from the chromatographic column was flow-split with approximately 10% entering the mass spectrometer source and 90% directed towards a fraction collector. Dead volume between column and fraction collector was minimal (20 μl). The molecular masses of polypeptides in each chromatographic fraction were further analysed using matrix-assisted laser desorption/ionization, time-of-flight mass spectrometry (MALDI-TOF MS) on a linear time-of-flight Voyager DE mass spectrometer (Perseptive Biosystems, MA, USA) in positive detection mode using alpha-cyano-4-hydroxycinnamic acid as the matrix. Internal mass calibration of the instrument with known standards established the accuracy of mass determination as ±0.1%. The peptides with masses coincident with those of dermatoxin and phylloxin as deduced from cloned precursor cDNAs were each subjected to primary structural analysis by automated Edman degradation using an Applied Biosystems 491 Procise sequencer

in pulsed-liquid mode or by MS/MS fragmentation sequencing using the LCQ™.

3. Results

3.1. "*Shotgun*" *cloning of P. sauvagei dermatoxin and phylloxin cDNAs*

Two novel peptide cDNAs were consistently cloned from the skin secretion library (sequencing of 25 clones, each sequence represented at least 10 times) and each encoded a single copy of novel dermatoxin (DRT-S) and phylloxin (PLX-S)-related peptides (Fig. 1). Open-reading frames consisted of 77 and 64 amino acid residues, respectively. The nucleotide sequences of dermatoxin (DRT-S) and phylloxin (PLX-S) have been deposited in the EMBL Nucleotide Sequence Database under the accession codes AJ865344 and AJ865345, respectively. An EBI-BLAST search found that DRT-S and PLX-S nucleotide sequences showed 90% and 94% sequence identity, respectively, with the dermatoxin and phylloxin sequences from *Phyllomedusa bicolor* [14,15] (Fig. 2). However the EBI-BLAST search

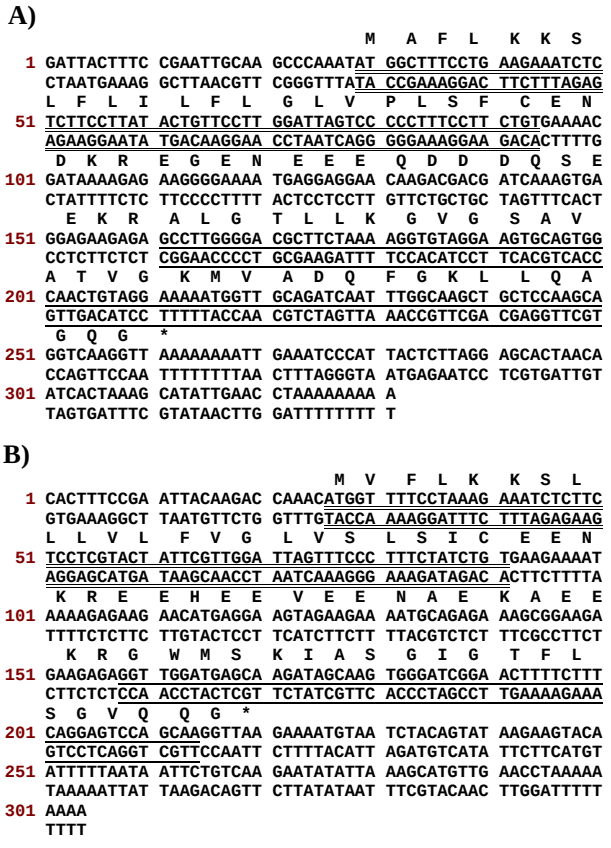

Fig. 1. Nucleotide sequences of precursor cDNAs encoding *Phyllomedusa sauvagei* dermatoxin (DRT-S) (A) and phylloxin (PLX-S) (B) cloned from a skin secretion-derived library. The putative signal peptides (double-underlined), mature processed peptides (single-underlined) and stop codons (asterisks) are indicated.

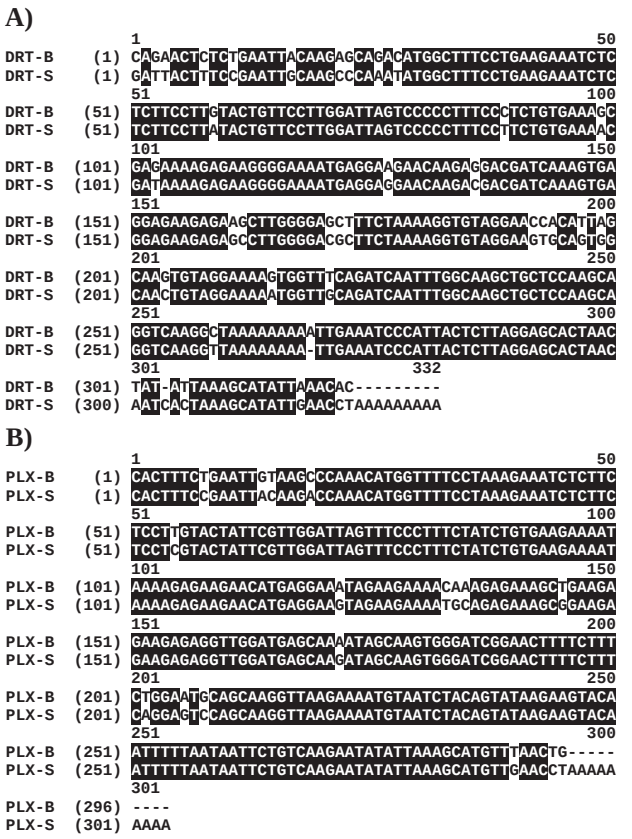

Fig. 2. Alignment of nucleotide sequences of dermatoxin precursors from *Phyllomedusa bicolor* (DRT-B) and *Phyllomedusa sauvagei* (DRT-S) (A) and corresponding phylloxin precursors (PLX-B and PLX-S) (B). Identical bases are shaded in black.

revealed that DRT-S and PLX-S open-reading frame amino acid sequences showed 81% and 95% sequence identity, respectively, with dermatoxin and phylloxin precursor sequences from *Phyllomedusa bicolor* [14,15] (Fig. 3). The conserved preproregion includes a putative 22 amino acid residue signal peptide followed by a 22 amino acid residue acidic peptide that terminates in a typical-Lys-Arg-(-KR-) propeptide convertase processing motif that is responsible for cleavage and release of each respective mature peptide located at the C-terminus.

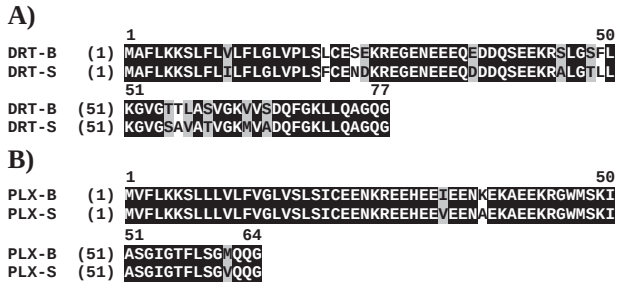

Fig. 3. Alignment of translated open-reading frame amino acid sequences of (A) dermatoxin precursors from *Phyllomedusa bicolor* (DRT-B) and *Phyllomedusa sauvagei* (DRT-S) and (B) corresponding phylloxin precursors (PLX-B and PLX-S). Identical amino acid residues are shaded in black and conservative residue substitutions in grey.

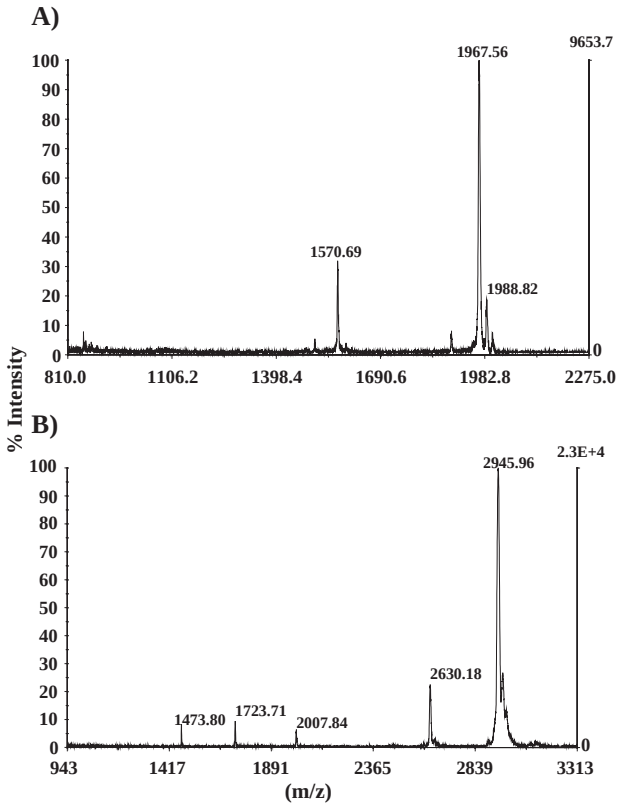

Fig. 4. MALDI-TOF mass spectra of *Phyllomedusa sauvagei* skin secretion chromatographic fractions containing (A) phylloxin (1967.56 Da-MH^+) (A) and (B) dermatoxin (2945.96 Da-MH^+).

3.2. Isolation and structural characterization of P. sauvagei dermatoxin and phylloxin

One novel dermatoxin (DRT-S), with a computed molecular mass of 2945.96 Da (ALGTLLKGVGSAVATVGKMVADQFGKLLQA-amide), and a novel phylloxin (PLX-S), with a computed molecular mass of 1967.56 Da (GWMSKIASGIGTFLSGVQQ-amide), were identified in chromatographic fraction numbers 127 and 132, respectively, and hence were completely resolved from one another by HPLC (Figs. 4 and 5). Their respective primary structures were confirmed by either automated Edman degradation or by MS/MS (data no shown). The NCBI-BLAST search found that DRT-S and PLX-S showed 70% and 94% sequence identity, respectively, with dermatoxin and phylloxin from *Phyllomedusa bicolor* [14,15]. An interesting observation was that phylloxin from *P. sauvagei* exhibited only a single site conservative amino acid substitution (Val for Met at position 17) when compared with phylloxin from *P. bicolor* [15]. In contrast, the primary structures of dermatoxin from the two species were less highly conserved exhibiting nine, chemically conservative amino acid substitutions. A further feature of interest was the absence of the C-terminal dipeptide, −GQ, on the *P. sauvagei* dermatoxin homolog.

4. Discussion

The defensive skin secretions of frogs are known to contain a plethora of biologically active peptides, some of which share common primary structural features with endogenous vertebrate regulatory peptides but others, often indeed the most abundant, are taxon-specific broad-spectrum antimicrobials such as bombinins, magainins, brevinins, dermaseptins [19]. The spectrum of microorganisms susceptible to skin secretions of the Phyllomedusinae is broad, probably due to the combined presence of a variety of antimicrobial peptides, which differ in primary structure from one another by only a few amino acid residues [16]. The dermaseptins are a family of broad spectrum antimicrobial peptides, 27–34 amino acid residues in chain length, which probably play a pivotal role in the defense of the naked skin of phyllomedusine frogs against microbial invasion. They are the first vertebrate peptides to show lethal effects against the filamentous fungi responsible for severe opportunistic infections accompanying immunodeficiency syndrome and following the use of immunosuppressive agents [20].

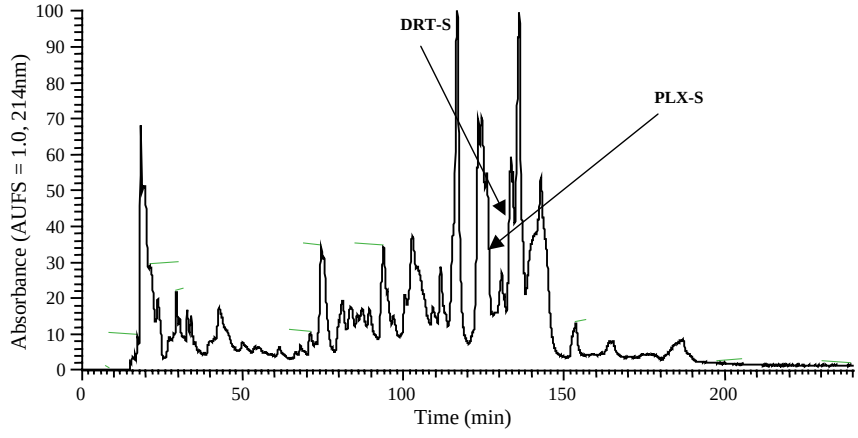

Fig. 5. Reverse phase HPLC chromatogram of *Phyllomedusa sauvagei* skin secretion. The absorbance peaks representing dermatoxin (DRT-S) and phylloxin (PLX-S) are indicated.

Unlike most, if not all, previously characterized dermaseptins, dermatoxin and phylloxin are two, relatively recently discovered phyllomedusine skin secretion peptides that exhibit a narrow, but nevertheless significant, spectrum of antimicrobial activity [14,15]. Interestingly, both peptides are particularly active against mollicutes (cell wall-less bacteria) while being highly selectively active against Gram +ve and Gram −ve bacteria [14,15]. Thus, the skin secretions of phyllomedusine frogs appear to contain a veritable pharmacopoeia of antimicrobial peptides that probably confer protection to the largest possible array of micro-organisms in a manner that is essentially combination therapy—a lesson that we humans have now learned as the best approach to prevent accumulated resistance.

While phylloxin was a truly novel prototype peptide, dermatoxin sequences were present in and predicted from cDNA clones generated previously from skin libraries of the phyllomedusine frogs, *Pachymedusa dacnicolor* and *Agalychnis annae* [21] (see Fig. 6). Although the peptides were predicted and not isolated and screened for antimicrobial activity, nevertheless these data provide an important insight. They prove that narrow activity spectrum antimicrobial peptides such as dermatoxin, are present in members of the two major known phyllomedusine genera, *Phyllomedusa* and *Agalychnis*, as well as in the sole representative of the monotypic genus, *Pachymedusa*. This fact, coupled with their high degree of primary structural conservation, would suggest that they represent discrete entities whose presence in the secretion is necessary in the mediation of an important biological function. Here we describe the structures of two novel peptides from *P. sauvagei*, a dermatoxin and a phylloxin, deduced in the first instance from "shotgun" cloned precursor cDNAs, and subsequently identified and structurally characterized from skin secretion. Both of these experimental procedures were carried out on the same sample of lyophilized skin secretion that was obtained in a non-invasive, non-lethal manner. Parallel data from the original studies on *P. bicolor*, required the sacrifice of two specimens [14,15]. Our current data have now unequivocally shown that such data can be generated without this requirement and without compromise of robustness and quality.

The *P. sauvagei* phylloxin and dermatoxin precursor structures, deduced from cloned cDNAs, share the same organization as their homologs from *P. bicolor*, which in turn reflect a highly conserved architecture shared by many antimicrobials from phyllomedusine frog skin [16]. This consists of a putative signal peptide of 20–23 amino acid residues, a typical propeptide convertase processing site (-KR-), an intervening acidic amino acid residue-rich spacer peptide, a second typical processing site and a terminal antimicrobial peptide-encoding domain. The latter represents the hypervariable domain. Of interest is the post-translational processing that occurs at the C-terminal region of the open-reading frame. In the case of dermatoxin from *P. bicolor*, the C-terminal glycyl residue of the precursor open-reading frame acts as an amide donor for the penultimate glutaminyl residue in contrast to *P. sauvagei*, where the glycyl residue at −3 from the C-terminus acts as an amide donor for the alanyl residue at position −4 that becomes the C-terminal feature of the mature peptide. Only a trace of the extended form was located in skin secretion fractions by mass spectrometry and this represented less than 0.2% of the alaninamide terminating peptide by ion abundance. It thus appears that differences in post-translational processing of highly structurally homologous peptide precursors can occur between very closely related amphibian species, in fact, we have previously demonstrated that this phenomenon can be demonstrated even within conserved dermaseptin precursors of single species such as *P. sauvagei* [22].

```
Phyllomedusa sauvagei     ALGTLLKGVGSAVATVGKMVADQFGKLLQAGQG
Phyllomedusa bicolor      SLGSFLKGVGTTLASVGKVVSDQFGKLLQAGQG
Pachymedusa dacnicolor    SLGSFMKGVGKGLATVGKIVADQFGKLLEAGQG
Agalychnis annae          SLGFSMKGVGKGLATVGKIVADQFGKLLEAGKG
                           **   ****   * *** * ******* ** *
```

Fig. 6. Comparison of aligned amino acid sequences of non post-translationally modified dermatoxin-encoding domains deduced from cloned precursor cDNAs of phyllomedusine frogs. Asterisks indicate amino acid residue conservation.

A significant feature of the present study regards the NCBI-BLAST search. The open reading frame nucleotide sequences of the novel peptide cDNAs encoding the putative signal peptide and the first three residues of the acidic spacer peptide domain, share about 50%–70% identity with the corresponding regions of antimicrobial peptide cDNAs from other amphibian groups. These include the caerin, esculentin, brevinin, temporin and gaegurin cDNAs. This finding reinforces the hypothesis that one exon encoding the signal peptide of several otherwise unrelated peptide precursors was present early in the evolution of the Amphibia. The duplication and recombinational events that promoted the association of such a homologous secretory exon with nucleotide sequences coding for a variety of end products in various amphibian species remain to be elucidated but are likely to have occurred at the very early stages of species radiation [23].

However, the structural and the related functional differences of amphibian skin antimicrobial peptides, even within a single species that possesses multiple isomeric forms of broad-spectrum actives such as dermaseptins, and smaller quantities of narrow-spectrum actives, such as dermatoxin and phylloxin, may be suggestive of a naturally selected mechanism directed towards generating maximum possible microbicidal effects while simultaneously minimizing generation of resistance mechanisms. This is in effect the natural application of combination therapy that we now know should provide both of these highly desirable endpoints most effectively. Such strategies, as have evolved in nature subject to aeons of selection pressures, should be more critically evaluated for therapeutic drug design and application.

Acknowledgement

Mei Zhou is currently in receipt of an Overseas Research Studentship from Queen's University, Belfast, Northern Ireland.

References

[1] Hancock REW, Falla T, Brown MH. Cationic bactericidal peptides. Adv Microb Physiol 1995;37:135–75.
[2] Gabay JE. Ubiquitous natural antibiotics. Science 1994;264:373–4.
[3] Nue HC. The crisis in antibiotic resistance. Science 1992;257: 1064–73.
[4] Kreil G. Antimicrobial peptides from amphibian skin: an overview. Ciba Found Symp 1994;186:77–90.
[5] Bevins C. Antimicrobial peptides as agents of mucosal immunity. Ciba Found Symp 1994;186:250–69.
[6] Boman HG. Peptide antibiotics and their role in innate immunity. Annu Rev Immunol 1995;13:61–92.
[7] Erspamer V. Bioactive secretions of the integument. In: Heatwole H, Barthalmus GT, editors. The integument. Amphibian Biology, vol. 1. Chipping Norton: Surrey Beatty & Sons; 1994. p. 179–350.
[8] Simmaco M, Mignogna G, Barra D. Antimicrobial peptides from amphibian skin: what do they tell us? Biopolymers 1998;47:435–50.
[9] Witkop B, Brossi A. Natural toxins and drug development. In: Krogsgaard-Larsen P, Brogger Christensen S, Kofod H, editors. Natural products and drug development. Alfred Benzon Symposium, vol. 20, Munksgaard, Copenhagen. p. 283–300.
[10] Clarke BT. The natural history of amphibian skin secretions, their normal functioning and potential medical applications. Biol Rev Camb Philos Soc 1997;72:365–79.
[11] Walls JG. Red-eyes and other leaf-frogs. Manufactured in the USA. T.F.H. Publications, Inc.
[12] Erspamer V, Falconieri Erspamer G, Cei JM. Active peptides in the skins of two hundred and thirty American amphibian species. Comp Biochem Physiol C 1986;85:125–37.
[13] Amiche M, Seon AA, Pierre TN, Nicolas P. The dermaseptin precursors: a protein family with a common preproregion and a variable C-terminal antimicrobial domain. FEBS Lett 1999;456(3): 352–6.
[14] Amiche M, Seon AA, Wroblewski H, Nicolas P. Isolation of dermatoxin from frog skin, an antibacterial peptide encoded by a novel member of the dermaseptin genes family. Eur J Biochem 2000;267:4583–92.
[15] Pierre TN, Seon AA, Amiche M, Nicolas P. Phylloxin, a novel peptide antibiotic of the dermaseptin family of antimicrobial/opioid peptide precursors. Eur J Biochem 2000;267:370–8.
[16] Vanhoye D, Bruston F, Nicolas P, Amiche M. Antimicrobial peptides from hylid and ranid frogs originated from a 150-million-year-old ancestral precursor with a conserved signal peptide but a hypermutable antimicrobial domain. Eur J Biochem 2003;270:2068–81.
[17] Chen T, Bjourson AJ, McClean S, Orr DF, O'Kane EJ, Rao P, et al. Cloning of maximakinin precursor cDNAs from Chinese toad, *Bombina maxima*, venom. Peptides 2003;24:853–61.
[18] Chen T, Farragher S, Bjourson AJ, Orr DF, Rao P, Shaw C. Granular gland transcriptomes in stimulated amphibian skin secretions. Biochem J 2003;371:125–30.
[19] Bevins CL, Zasloff M. Peptides from frog skin. Ann Rev Biochem 1990;59:395–414.
[20] Amiche M, Ducoucel F, Boulain JC, Menez A, Nicolas P. Precursors of vertebrate peptide antibiotics dermaseptin b and adenoregulin have extensive sequence identities with precursors of opioid peptides dermorphin, dermenkephalin, and deltorphins. J Biol Chem 1994;269:17847–52.
[21] Wechselberger C. Cloning of cDNAs encoding new peptides of the dermaseptin-family. Biochim Biophys Acta 1998;1388:279–83.
[22] Chen T, Tang L, Shaw C. Identification of three novel *Phyllomedusa sauvagei* dermaseptins (sVI-sVIII) by cloning from a skin secretion-derived cDNA library. Regul Pept 2003;116:147–54.
[23] Charpentier S, Amiche M, Mester J, Vouille V, Le Caer JP, Delfour A, et al. Structure, synthesis, and molecular cloning of dermaseptins B, a family of skin peptide antibiotics. J Biol Chem 1998;273:14690–7.