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ORIGINAL ARTICLE

Vipericidins: a novel family of cathelicidin-related peptides from the venom gland of South American pit vipers

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Abstract Cathelicidins are phylogenetically ancient, pleiotropic host defense peptides—also called antimicrobial peptides (AMPs)—expressed in numerous life forms for innate immunity. Since even the jawless hagfish expresses cathelicidins, these genetically encoded host defense peptides are at least 400 million years old. More recently, cathelicidins with varying antipathogenic activities and cytotoxicities were discovered in the venoms of poisonous snakes; for these creatures, cathelicidins may also serve as weapons against prey and predators, as well as for innate immunity. We report herein the expression of orthologous cathelicidin genes in the venoms of four different South American pit vipers (*Bothrops atrox*, *Bothrops lutzi*, *Crotalus durissus terrificus*, and *Lachesis muta rhombeata*)—distant relatives of Asian cobras and kraits, previously shown to express cathelicidins—and an elapid, *Pseudonaja textilis*. We identified six novel, genetically encoded peptides: four from pit vipers, collectively named vipericidins, and two from the elapid. These new venom-derived cathelicidins exhibited potent killing activity against a number of bacterial strains (*S. pyogenes, A. baumannii, E. faecalis, S. aureus*, *E. coli*, *K. pneumoniae,* and *P. aeruginosa*), mostly with relatively less potent hemolysis, indicating their possible usefulness as lead structures for the development of new anti-infective agents. It is worth noting that these South American snake venom peptides are comparable in cytotoxicity (e.g., hemolysis) to human cathelicidin LL-37, and much lower than other membrane-active peptides such as mastoparan 7 and melittin from bee venom. Overall, the excellent bactericidal profile of vipericidins suggests they are a promising template for the development of broad-spectrum peptide antibiotics.

Keywords Venom peptides · Antimicrobial peptides · Reptilian CRAMPs · Vipericidins · Peptide synthesis · Therapeutic peptide

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Introduction

In the fight against infectious diseases, the alarming emergence rates of multidrug-resistant microorganisms and the ensuing need to treat recurrent infections place increasing pressure on developing novel, more effective and selective antibiotics (Brusselaers et al. 2011). In this regard, antimicrobial peptides (AMPs) constitute an increasingly recognized alternative to conventional antibiotics. The innate immune system of vertebrates, acting as first line of immediate defense against pathogenic organisms, features AMPs as key components, selected in the course of evolution for their ability to target bacteria, fungi, protozoa,

and viruses (Torrent et al. 2013a, b; Maróti et al. 2011; Cole and Lehrer 2003; Zasloff 2002; Andreu and Rivas 1998). In contrast to chemical antibiotics of microbial origin, produced by sequential enzymatic pathways, eukaryotic AMPs originate from gene-encoded precursors (Zhu and Gao 2013; Mangoni 2011; Tossi and Sandri 2002; Zanetti et al. 1995) that give rise to mature forms usually not larger than 60 amino acid residues and structurally classified as linear (α-helical and non-helical), cyclic (θ-defensins), and cross-linked by variable numbers of internal disulfide bridges. In terms of amino acid composition and net surface charge, AMPs can present enrichment in certain amino acid residues (i.e., proline, glycine, tryptophan, histidine, lysine, or arginine) conferring them variable levels of hydrophobicity, amphipathicity, net positive or, less frequently, net negative charge (Zasloff 2002; Tossi and Sandri 2002). Up to now, over a thousand AMP sequences have been described from different phyla and species, as listed in various dedicated databases (http://aps.unmc.edu/AP/main.php; http://apps.sanbi.ac.za/dampd/; http://biotechlab.fudan.edu.cn/database/lamp, Table S1).

Among AMPs involved in human host defense, the cathelicidin family is one found mainly in the granules of circulating neutrophils and in macrophages at strategic sites such as skin and mucosal surfaces, and also produced by epithelial cells, mast cells, and other cell types, including glia (Brandenburg et al. 2008). Cathelicidins derive from a four-exon/three-intron gene that is processed onto a precursor with an *N*-terminal domain containing both a signal peptide and a proregion sequence, followed by the mature peptide at the *C*-terminus. The name cathelicidin derives from the characteristic sequence homology between the proregion of this AMP family and cathelin, a cathepsin L (cysteine proteinase) inhibitor. The mature cathelicidin is released from the cathelin-like proregion during the process of degranulation occurring inside a phagocytic vacuole or in an extracellular environment (Bals and Wilson 2003; Tomasinsig and Zanetti 2005). Based on amino acid sequence, three major types of mature cathelicidins have been distinguished among different species: linear structures folding into α-helices upon interaction with biological membranes; sequences with cysteine pairs that fold into hairpin-like motifs, and peptides with a high proportion of one or two amino acids, such as proline and tryptophan. Humans express a single cathelicidin, of the helical class, called LL-37 (Dürr et al. 2006). Despite a structural diversity among species, all cathelicidins display typical physico-chemical features of AMPs; and LL-37 plays diverse interconnected biological roles in the physiology of human tissues (Bals and Wilson 2003; Tomasinsig and Zanetti 2005; Wong et al. 2013). Recent research has shown that cathelicidins and their complexes with DNA or other biomolecules are implicated in the development or exacerbation of severe skin diseases such as systemic lupus erythematosus and psoriasis, as well as the inflammatory damage of tissue in arthritis (Morizane and Gallo 2012; Yamasaki and Gallo 2011; Hoffmann et al. 2013).

Initially identified in bovine neutrophils (Bac5) (Zanetti et al. 1990), species-related cathelicidins were later found in pigs (PR-39, protegrins, etc.), sheep (SMAPs, etc.), goats (ChBac5), horses (eCATHs), rabbits (CAP18), guinea pigs (CAP11), rats (rCRAMP), mice (mCRAMP), and monkeys (RL-37). The numbers and types of different cathelicidins found in mammals vary; some species have up to a dozen, while others have only one, reflecting different selective pressures from pathogens, host tissues, and the environment (Zasloff 2002; Tomasinsig and Zanetti 2005). As mentioned above, in humans, the only cathelicidin identified is LL-37, a peptide expressed in a variety of tissues including the skin, testes, gastrointestinal, and respiratory tract (Frohm et al. 1997; Bals and Wilson 2003; Zaiou and Gallo 2002; Ramanathan et al. 2002) as well as the brain (Brandenburg et al. 2008). Cathelicidins are also found in birds (Lynn et al. 2004; Xiao et al. 2006), amphibians (Hao et al. 2012) and fish (Chang et al. 2006), even in living fossils like the jawless craniate hagfish (Uzzell et al. 2003). The presence of cathelicidins in such primitive vertebrates indicates that cathelicidin genes originated early in the course of biological evolution, and have been maintained in the genomes of all vertebrates.

In reptiles, cathelicidin-related sequences were purified and cloned from the venoms of three Asian elapid snake species (Wang et al. 2008; Zhao et al. 2008). BF-30 cathelicidin from *Bungarus fasciatus* (banded krait) is a potent, broad-spectrum membranolytic AMP with antitumor activity (Wang et al. 2008, 2013). Cathelicidin-related antimicrobial peptides (CRAMPs) from *Naja atra* (Na_CRAMP, Chinese cobra) and *Ophiophagus hannah* (Oh_CRAMP, king cobra) are structurally very similar to BF-30 cathelicidin and display antimicrobial activity against wide range of bacteria even in the presence of high concentrations of salt (Zhao et al. 2008).

It is intriguing that cathelicidins are utilized as weapons against prey and predators in snake venom, and also deployed in the granules of mammalian white blood cells and epithelial tissues to control pathogens and infected or cancerous cells. In both cases, it seems that the strong toxicities of cathelicidins towards certain cells are being exploited to the host's advantage, yet in very different ways. Given the wide distribution of cathelicidin-related peptides and genes among vertebrate species, including very primitive forms like hagfish and snakes like cobras and kraits, we have herein investigated the expression of orthologous genes in the venoms of four South American pit vipers—distant relatives of Asian cobras and kraits—and one elapid

in addition, and identified six encoded novel peptides (four from pit vipers, collectively named vipericidins; two from the elapid). These venom-derived cathelicidins are found to have potent antimicrobial activity against Gram-negative bacteria, with low cytotoxicity, thus making these novel peptides good candidates as antimicrobial agents. Vipericidins and their closely related elapid CRAMPs constitute a new addition to the already substantial number of animal toxin-derived scaffolds for the development of novel therapeutic molecules (Rocha e Silva et al. 1949; Sabatier 2011; Zaenker 2011; Cury and Picolo 2006; Mirshafiey 2007; Mortari et al. 2007; Watters 2005), which in either naïve or engineered versions are at various phases of clinical and diagnostic applications (Fox and Serrano 2007; Lewis and Garcia 2003).

Materials and methods

cDNA libraries from South American pit viper and elapid venom glands

Four species belonging to three genus of South American pit vipers (Crotalinae subfamily) were investigated for cathelicidin-like precursors, namely *Bothrops atrox*, *Bothrops lutzi*, *Crotalus durissus terrificus*, and *Lachesis muta rhombeata* (the latter an endangered species). Surgery to remove the venom glands and molecular biology procedures to prepare cDNAs were as previously reported (Radis-Baptista 2011). The venom gland from the elapid *Pseudonaja textilis* was a gift from Dr Patrick J. Spencer (Biotechnology Center, Nuclear and Energy Research Institute—IPEN/CNEN, São Paulo, Brazil). The five independent pools of venom gland cDNAs were searched by PCR homology screening using the primers in Table S2 (IDT DNA Technologies, Coralville, IA, USA). Screening involved a touchdown (TD-)PCR consisting of a denaturing step (95 °C for 3 min), 30 annealing and elongation cycles (95 °C for 30 s, 60 °C for 30 s, −1 °C per cycle in the first ten cycles, and 72 °C for 2 min), and a final step of 72 °C for 8 min to polish the amplicon ends. Amplicons were cloned into pGEM-T Easy Vector System (Promega, Madison-WI, USA), and plasmids corresponding to positive clones were prepared for sequencing according to standard procedures (Sambrook and Russel 2011).

Sanger sequencing, gene ontology and functional annotation

Putative cathelicidin-like cDNAs were sequenced by the dideoxy chain termination (Sanger) method using universal primers T7 and SP6 and the BigDye Terminator v3.1 Cycle Sequencing Kit in a 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA). Good quality DNA sequences (Phred scores >20) were used to search non-redundant gene and protein database, by means of 'nucleotide blast' and 'blastx' (http://blast.ncbi.nlm.nih.gov/) search tools. The best 100 hits, including the prepropeptide sequences of mammalian cathelicidins and other animal cathelicidin-related sequences, were combined in a single file and used for gene ontology analyses and manual sequence annotation. Molecular phylogenetic relationships were established by multi-aligning the deduced amino acid sequences with the clustalw2 program (http://www.ebi.ac.uk/Tools/clustalw2), and the phylogenetic tree inferred using default parameters. The annotated vipericidins and elapid CRAMP sequences were submitted to GenBank (http://www.ncbi.nlm.nih.gov/), with accession numbers JX948107 to JX948115.

qPCR analysis of CRAMP gene expression in the venom glands

CRAMP expression levels in the venom glands of the four species of South American pit vipers and the elapid *P. textilis* were studied by quantitative real-time polymerase chain reaction. Based on the sequences of the 5′- and 3′-ends of multi-aligned CRAMPs, consensus primers (Table S2) were designed and synthesized. Crotalicidin from *C. durissus terrificus* served as template for a standard curve constructed by 10-fold serial dilution of a cDNA solution. Analysis of reptilian cathelicidin-like expression level was carried out in the Rotor Gene 3000 operated by its specific software, version 6.0.19 (Corbett Research, Mortlake, Australia). Each reaction consisted of 2 µl of single-stranded (ss)-cDNA, 10 µl of SYBR Green PCR Core Reagent (Applied Biosystems, Foster City-CA, USA) and primers to give a 200 nM concentration in a final reaction volume of 20 µl. Amplification conditions involved 45 cycles at 95 °C for 30 s, at 55 °C for 20 s, and at 72 °C for 20 s. Fluorescence was monitored (494–521 nm) during the extension phase. Specificity of each reaction was assessed by a melting curve (55–90 °C; 1 °C/5 s) and the melting temperature (T_m) used to discriminate CRAMP cDNA precursors. Threshold and threshold cycle (C_t) values were automatically determined by the Rotor Gene software using default parameters. The C_t and T_m data were expressed as mean of two or more measurements ± SEM. For absolute quantification of CRAMP gene products, the following equation (http://cels.uri.edu/gsc/cndna.html) relating the number of transcript molecules to DNA amount, length and mean nucleotide MW was applied:

$$\text{number of transcripts} = (\text{DNA amount} \times 6.022 \times 10^{23})/(\text{length} \times 1 \times 10^{9} \times 650).$$

The transcript copy numbers used were a mean of nine measurements ± SEM, and were normalized against those

of snake β-actin in the venom gland tissue. The corresponding real-time PCR efficiencies (E) were calculated from the slopes (S) given by the Rotor Gene software, according to the equation: $E = 10^{(-1/k)} - 1$. Linearity was expressed as the square of the Pearson correlation coefficient (R^2).

Prediction of antimicrobial regions in snake venom CRAMPs

The six novel putative CRAMP sequences derived from South American pit viper and elapid venoms were analyzed by AMPA (Torrent et al. 2012), a predictive algorithm for identification of peptide stretches with antimicrobial properties (Fig. S2). In all cases, regions immediately downstream from the cathelin domain displayed scores indicative of significant antimicrobial activity.

Synthetic versions of the predicted CRAMPs

Solid phase syntheses of the vipericidins crotalicidin and batroxicidin, and of elapid CRAMPs from *P. textilis* (Pt_CRAMP1) and *O. hanna* (Oh_CRAMP), were performed in a Prelude peptide synthesizer (Protein Technologies, Tucson, AZ, USA) running Fmoc protocols at 0.05-mmol scale on Fmoc-Rink-amide ChemMatrix resin. Side chain functionalities were protected with tert-butyl (Glu, Ser, Thr, Tyr), N^G-2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Arg), and trityl (Cys) groups. Eightfold excess of Fmoc-L-amino acids and HBTU, in the presence of a double molar amount of DIEA, were used for the coupling steps, with DMF as solvent. After chain assembly, full deprotection and cleavage were carried out with TFA/H_2O/TIS (95:2.5:2.5 v/v, 90 min, rt). Peptides were precipitated by the addition of chilled diethyl ether, taken up in aqueous HOAc (0.1 M) and lyophilized. Analytical reversed-phase HPLC was performed on a Luna C18 column (4.6 × 50 mm, 3 μm; Phenomenex, Jupiter, CA, USA). Linear gradients of solvent B (0.036 % TFA in ACN) into A (0.045 % TFA in H_2O) were used for elution at a flow rate of 1 mL/min, and with UV detection at 220 nm. Preparative HPLC runs were performed on a Luna C18 column (21.2 × 250 mm, 10 μm; Phenomenex, Jupiter, CA, USA), using linear gradients of solvent B (0.1 % in ACN) into A (0.1 % TFA in H_2O), as required, with a flow rate of 25 mL/min. LC–MS was performed in a LC–MS 2010EV instrument (Shimadzu) fitted with an XBridge column (4.6 × 150 mm, 3.5 μm, Waters, Cerdanyola del Vallès, Spain) eluted with $A = 0.1$ % formic acid in water; $B = 0.08$ % formic acid in acetonitrile in gradients over 15 min at a flow rate of 1 mL/min, with UV detection at 220 nm. Fractions of adequate HPLC homogeneity and with the expected mass were combined, lyophilized and used in the experimental assays.

Antimicrobial assay

Assays were performed on clinical strains of *Streptococcus pyogenes* and *Acinetobacter baumannii* (from the laboratory of Prof. Jordi Vila, Department of Clinical Microbiology, Hospital Clínic, University of Barcelona) and on reference strains of *Enterococcus faecalis* (ATCC 29212), *Staphylococcus aureus* (ATCC 29213), *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 600703), and *Pseudomonas aeruginosa* (ATCC 27853). Gentamycin—an antibiotic that inhibits protein synthesis in both Gram-negative and Gram-positive bacteria—was used as control. Isolates were taken from the freezer and transferred at least twice on sheep blood agar to ensure purity and good growth. Strains were incubated for 24 h prior to testing. Inocula were prepared by direct suspensions of cells into saline to achieve the turbidity of the 0.5 McFarland standard. Minimal inhibitory concentration (MIC) was determined by the microdilution method in Mueller–Hinton broth after incubation at 35 °C for 24 h according to Clinical and Laboratory Standards Institute guidelines (CLSI 2012). For *S. pyogenes,* the Mueller–Hinton broth was supplemented with lysed horse blood (5 %).

Hemolytic activity

Fresh blood (10 mL) was collected in EDTA tubes and centrifuged at 1,000*g* for 10 min at 4 °C. After plasma removal, the pellet containing red blood cells (RBCs) was washed three times with PBS (35 mM phosphate, 150 mM NaCl, pH 7.4) and re-suspended in PBS to 8 % (v/v) RBC concentration. 100 μL of the suspension was added to microfuge tubes containing 100 μL of serially diluted (0.2–200 μM) peptides, to give final concentrations of 4 % (v/v) RBCs and 0.1–100 μM peptide. After incubation with gentle shaking at 37 °C for 30 min, samples were centrifuged at 1,000*g* for 2 min, supernatants were transferred to 96-well plates and released hemoglobin was determined spectrophotometrically at 540 nm in an Infinite® 200 (Tecan, Männedorf, Switzerland) reader. Triton X-100 at 1 and 4 % (v/v) RBCs in PBS with no peptide added (untreated) were used as positive and negative controls, respectively. Percentage of hemolysis was determined as $[OD_{540(treated)} - OD_{540(untreated)}]/[OD_{540(Triton)} - OD_{540(untreated)}] \times 100$. Experiments were carried out in triplicate.

Results

Cathelicidin-related peptides from the venom gland of pit vipers and elapids

cDNAs from four species of South American pit vipers (*B. atrox*, *B. lutzi*, *C. durisssus terrificus*, *L. muta rhombeata*)

Table 1 Names and sizes of reptilian CRAMPs from pit vipers and elapids

Peptide name	Source	ORF size (bp)	Peptide length		Reference
			Prepro	Mature	
Pit vipers					
Crotalicidin	*Crotalus durissus terrificus*	585	194	34	This study
Lachesicidin	*Lachesis muta rhombeata*	585	194	34	This study
Batroxicidin	*Bothrops atrox*	570	189	34	This study
Lutzicidin	*Bothrops lutzi*	570	189	34	This study
Elapids					
Bf_CRAMP	*Bungarus fasciatus*	576	191	34	Wang et al. (2008)
Oh_CRAMP	*Ophiophagus hannah*	576	191	34	Zhao et al. (2008)
Na_CRAMP	*Naja atra*	576	191	34	Zhao et al. (2008)
Pt_CRAMP1[a]	*Pseudonaja textilis*	555	184	34	This study
Pt_CRAMP2[a]	*Pseudonaja textilis*	555	184	34	This study

[a] Two different clones derived from the venom gland, with minimal primary structure differences; see Table 2

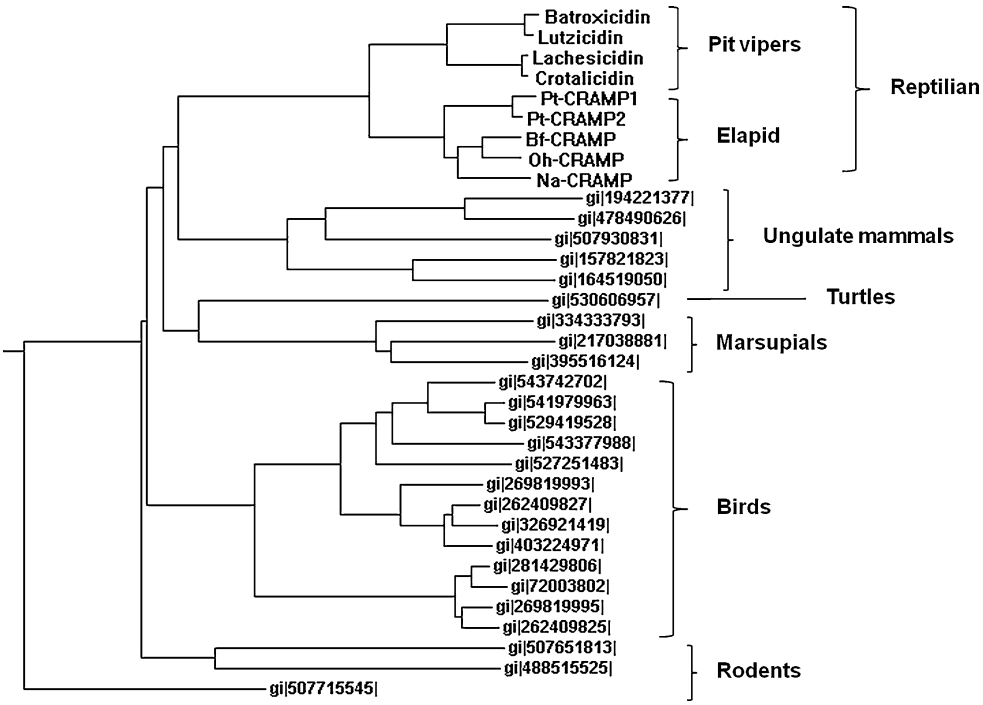

Fig. 1 Molecular phylogenetic analysis of 100-plus cathelicidin-related prepropeptides using ClustalW multi-alignment and tree building. Vipericidin and elapid sequences are nested within a clade that includes similar CRAMP sequences from birds, turtles, ungulate mammals, marsupials, and primitive rodents. Members of the reptilian branch are identified by their respective names; other vertebrate sequences by the corresponding gene identification (*GI*) numbers

and one elapid (cobra, *P. textilis*) species were cloned and sequenced. The 570–585 nucleotide (from start to stop codons) sequences encoded for 189–194 residue peptides (Table 1). Comparison of these polypeptide sequences against a non-redundant database retrieved 100 homologs—including hypothetical protein precursors—from the cathelicidin family, with scores ranging from $3E-61$ (100 % of coverage) to $4E-10$ (32 %). A phylogenetic tree (Fig. S1) built after clustalW multi-alignment (neighbor joining method) of hundreds of prepropeptide cathelicidin sequences shows the reptilian (snake venom) clade nested within a larger clade that includes turtles, birds, primitive rodents, marsupials, and ungulate mammals (Fig. 1).

The multi-alignment of the vipericidins (crotalicidin, lachesicidin, batroxicidin and lutzicidin) with other CRAMPs from Asian krait (*B. fasciatus*) and cobra (*O. hannah*, *N. atra* and *P. textilis*) shows a high structural conservation in both groups (Fig. 2), despite the evolutionary and geographical distance. Like other snake venom CRAMPs, the primary structures of the six peptides in this work comprise (in *N*- to *C*-terminal direction) the predicted signal peptide (ca. 20 residues), followed by a highly conserved cathelin-like domain folded by two disulfide bonds, and by a C-terminal region of varying sequence and size. This *C*-terminal region consists of a hypervariable (25–37 residues, Glu-rich) anionic moiety followed by a 34-residue, strongly cationic (Lys/Arg–rich)

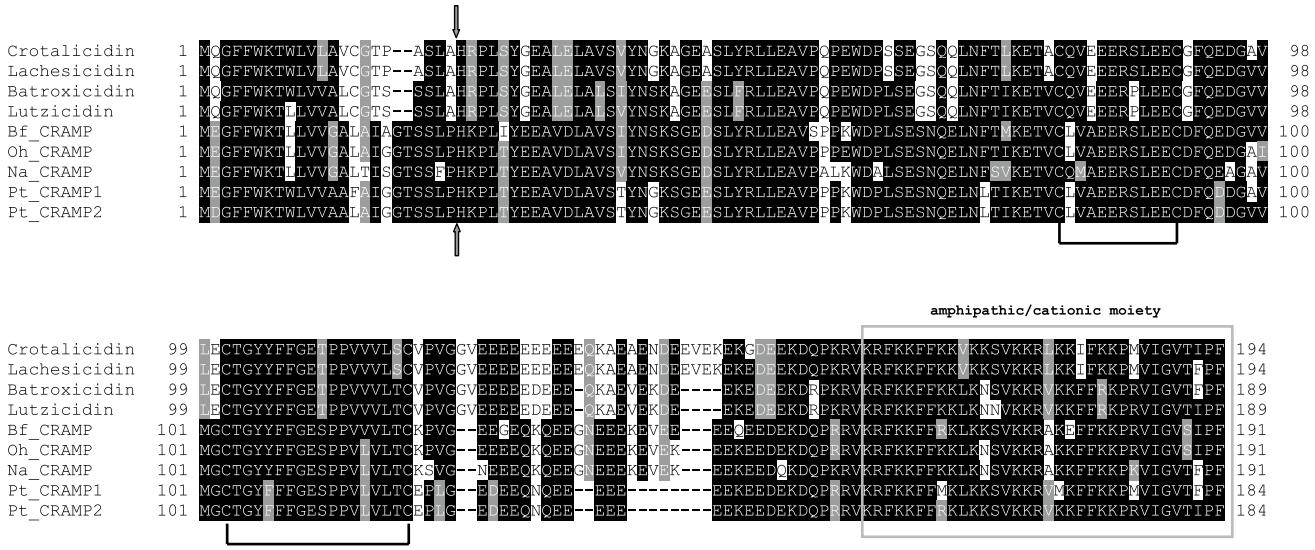

Fig. 2 Primary structures of South American pit viper and elapid venom gland CRAMP precursors. Sequences retrieved from protein and DNA databases were multi-aligned with ClustalW. Identical amino acid residues are highlighted in *black*, conservative replacements in *gray*. *Arrows* indicate the start of the cathelicidin domain, with its two conserved disulfide *bonds* also shown. The C-terminal region consists of a hypervariable anionic (Glu-rich) stretch followed by a 45 residue strongly basic final section where the antimicrobial activity resides

end section where the antimicrobial activity is confined (Fig. 2, open box; see below). Detailed sequence comparison of the C-termini of these snake venom CRAMPs with those of other clade members (turtles, birds, etc.) (Fig. 3) reveals the hypervariable anionic section preceding the cationic C-terminus as a characteristic feature of these reptilian CRAMPs.

CRAMPs are transcriptionally expressed in snake venom glands at various levels

To quantify the expression of CRAMP transcripts in the venom glands of the four South American pit viper species and the elapid *P. textilis*, rt-qPCR was used. Data are presented in Fig. 4 as the copy number normalized to 1 μg of β-actin (a constitutive housekeeping gene). Statistically, expression levels in the venom glands followed the trend *C. durissus terrificus* > *B. atrox* > *B. lutzi* > *L. muta rhombeata* ~ *P. textilis*. Regardless of these differences, which are discussed below, qPCR results confirm that all four vipericidins and the *P. textilis* CRAMP are actually transcribed and expressed in the snake venom gland.

The cationic/amphipathic, antimicrobially active end section of snake venom CRAMPs

Multi-alignment comparison of the strongly cationic C-termini of the peptide sequences of the present work with those of other snake venom CRAMPs (Fig. 2) clearly suggests these regions as potentially possessing antimicrobial activity. Further support can be obtained by running the six sequences on the AMPA algorithm, which reliably predicts antimicrobial peptide domains in proteins (Torrent et al. 2013a). AMPA profiles for the C-terminal sequences of *B. atrox*, *B. lutzi*, *C. durissus terrificus*, *L. muta rhombeata* (pit viper), and *P. textilis* (cobra) peptides (Fig. S2, panel A) show for all six of them a rather long (30–35 residue) well-defined stretch with propensity well below the 0.225 threshold and remarkable similarity to the profiles from Asian snake CRAMPs, hence strongly indicative of antimicrobial activity (Fig. S2). Experimental validation of these hints was obtained by means of synthetic versions of batroxicidin, crotalicidin and Pt_CRAMP1 which, together with Asian snake-derived Oh_CRAMP used as reference, were readily made by Fmoc solid phase synthesis, purified to >95 % homogeneity by HPLC and satisfactorily characterized by MS. In Table 2, the physico-chemical properties of reptilian CRAMPs in our present study are summarized. The antimicrobial activity of the four peptides was assayed against a panel of four standard and seven clinical Gram-negative and -positive bacterial strains. Data in Table 3 show that the South American snake venom peptides performed altogether rather effectively as antimicrobials against most organisms, with MICs in the low micromolar-to-submicromolar range (vs. clinical and standard strains, respectively). Activity was substantially higher against Gram-negative than -positive bacteria. Thus, for clinical Gram-negative strains, low-micromolar MICs were found for both crotalicidin and batroxicidin (as well as for the Oh_CRAMP reference), with a worth-noting

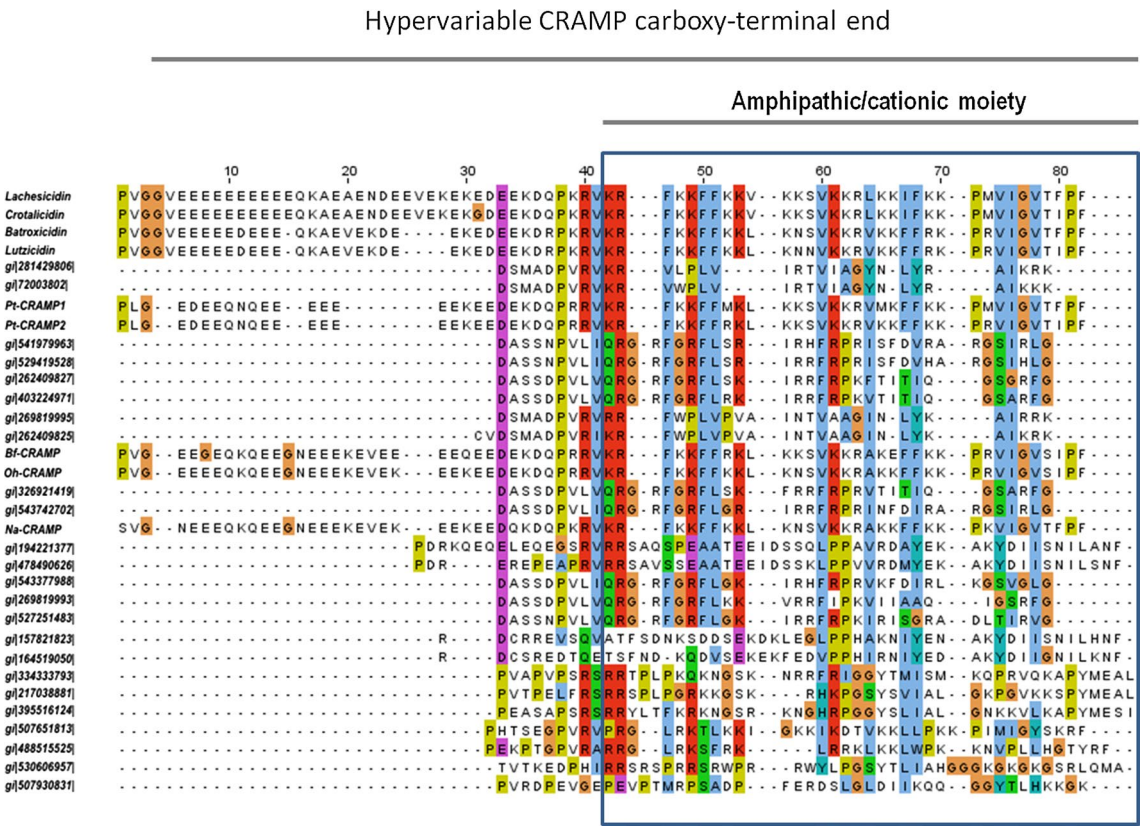

Fig. 3 Sequence analysis of snake venom CRAMPs shows the hypervariable anionic stretch preceding the C-terminus as a distinctive structural feature of the reptilian peptides compared to others from the same vertebrate clade

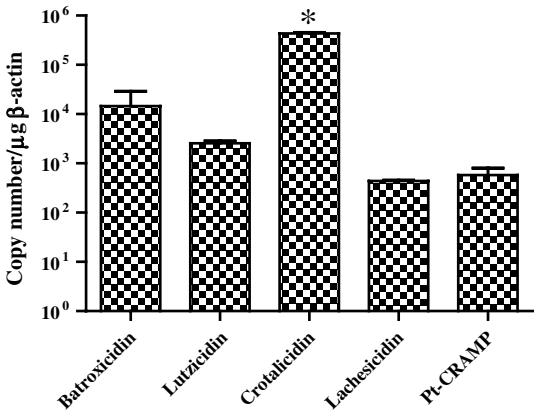

Fig. 4 Quantitative expression level of vipericidin transcripts in the venom gland of four pit vipers and one cobra species.*$P < 0.001$, one-way ANOVA and Bonferroni post hoc test

MIC of 1.9 µM for a clinical isolate of *K. pneumoniae.* As expected, these rather low clinical MICs dropped 1-to-2 log units into the submicromolar range for the corresponding ATCC standard strains. In contrast, Gram-positive strains required medium-to-high micromolar concentrations for inhibition, except for the rather sensitive *S. pyogenes* (MIC 3.8 µM). The Pt_CRAMP1 elapid-derived peptide was altogether marginally less potent than the two vipericidins and its Asian Oh_CRAMP counterpart. Against ATCC strains, it consistently performed ~1 log unit below the other three although, remarkably, in this case the differences between Gram-negative and -positive organisms were less acute.

Hemolytic activity

The hemolytic activity of the above four peptides was tested as a gauge of cytotoxicity. Results in Fig. 5 show the Asian elapid peptide Oh_CRAMP as the one with the lowest toxicity (10 % hemolysis at 100 µM) to erythrocytes. The vipericidins crotalicidin and batroxicidin were slightly more toxic, reaching 10 % hemolysis at 25 and 12.5 µM, respectively. Even so, extrapolation of Fig. 5 data indicates that 50 % hemolysis would be reached for all three peptides at near-millimolar values, which defines an adequate (~2 log) selectivity window. For the elapid-derived Pt_CRAMP1, and again in contrast with its Asian homolog, the poorer antimicrobial profile observed above was again accompanied by a rather strong hemolytic activity (10 % at ~6.25 µM and 50 % at only 50 µM), making it quite unselective.

Table 2 Sequence and physico-chemical properties of pit viper and elapid CRAMPs

Name	Sequence of mature reptilian CRAMPs[c]	Net charge[d]	pI[e]	Hydrophobic Index[f]	Molecular weight	
					Theory	Found
Batroxicidin[a]	KRFKKFFKKLKNSVKKRVKKFFRKPRVIGVTFPF	16	12.50	0.207	4,258.35	4,258.63
Crotalicidin[a]	KRFKKFFKKVKKSVKKRLKKIFKKPMVIGVTIPF	16	12.09	0.263	4,151.38	4,151.41
Lachesicidin[a]	KRFKKFFKKVKKSVKKRLKKIFKKPMVIGVTFPF	16	12.09	0.262	4,185.40	n.d.[g]
Lutzicidin[a]	KRFKKFFKKLKNNVKKRVKKFFRKPRVIGVTIPF	16	12.50	0.191	4,251.36	n.d.[g]
Pt_CRAMP1[b]	KRFKKFFMKLKKSVKKRVMKFFKKPMVIGVTFPF	14	12.08	0.357	4,225.46	4225.29
Pt_CRAMP2[b]	KRFKKFFRKLKKSVKKRVKKFFKKPRVIGVTIPF	16	12.50	0.196	4,238.41	n.d.[g]
Oh_CRAMP[b]	KRFKKFFKKLKNSVKKRAKKFFKKPRVIGVSIPF	16	12.34	0.172	4,154.24	4,154.11
Bf_CRAMP[b]	KRFKKFFRKLKKSVKKRAKEFFKKPRVIGVSIPF	15	12.04	0.171	4,197.27	n.d.[g]
Na_CRAMP[b]	KRFKKFFKKLKNSVKKRAKKFFKKPKVIGVTFPF	16	12.09	0.181	4,174.27	n.d.[g]

[a] From South American pit viper venoms

[b] From South American (first two) and Asian elapid venoms; Asian entries (Wang et al. 2008; Zhao et al. 2008) shown for comparison; Oh_CRAMP was synthesized as a cross-reference between CRAMPs from different sources and geographic areas

[c] Peptides have a C-terminal amide group

[d] Determined from http://www.innovagen.se/custom-peptide-synthesis/peptide-property-calculator/

[e] Determined by the compute_pi algorithm (http://web.expasy.org/compute_pi/)

[f] Determined by the Heliquest server (http://heliquest.ipmc.cnrs.fr/)

[g] As peptides were highly homologous to batroxicidin and crotalicidin, their synthesis was not judged necessary

Table 3 Minimal inhibitory concentration (MIC) of pit viper and elapid CRAMPs against standard and clinical bacterial strains

Microorganism	Gram	MIC, μg/mL (μM)				
		Crotalicidin	Batroxicidin	Pt_CRAMP1	Oh_CRAMP	Gentamicin
E. coli ATCC 25922	–	0.25 (0.06)	0.25 (0.05)	2 (0.5)	0.25 (0.06)	0.5 (1.0)
P. aeruginosa ATCC 27853	–	1 (0.2)	1 (0.2)	8 (1.9)	0.5 (0.1)	1 (2.1)
E. faecalis ATCC 29212	+	32 (7.7)	32 (7.5)	32 (7.6)	64 (15.4)	8 (16.8)
S. aureus ATCC 29213	+	32 (7.7)	32 (7.5)	32 (7.6)	64 (15.4)	0.25 (0.5)
E. coli	–	16 (3.8)	16 (3.8)	16 (3.8)	16 (3.8)	16 (33.5)
P. aeruginosa	–	16 (3.8)	16 (3.8)	32 (7.6)	16 (3.8)	8 (16.8)
K. pneumoniae	–	8 (1.9)	8 (1.9)	32 (7.6)	8 (1.9)	2 (4.2)
A. baumannii	–	16 (3.8)	16 (3.8)	16 (3.8)	16 (3.8)	8 (16.8)
E. faecalis	+	128 (30.8)	128 (30.0)	64 (15.1)	>128	16 (33.5)
S. aureus	+	32 (7.7)	32 (7.5)	64 (15.1)	32 (7.7)	0.25 (0.5)
S. pyogenes	+	16 (3.8)	16 (3.8)	16 (3.8)	16 (3.8)	8 (16.8)

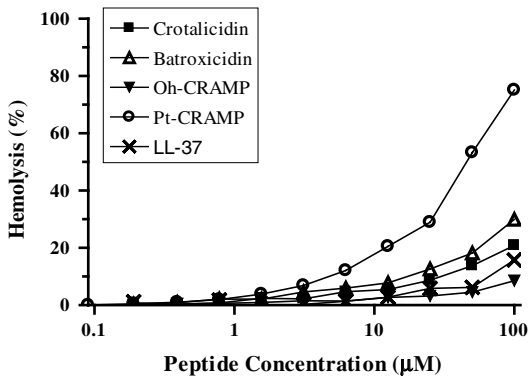

Fig. 5 Hemolytic activity of vipericidins (crotalicidin and batroxicidin) and cathelicidin-related peptides from Asiatic snakes

Discussion

The innate immune systems of vertebrates are equipped with diverse families of gene-encoded AMPs that include α-defensins, β-defensins, θ-defensin, and cathelicidins (Jenssen et al. 2006). The genes of such families appeared early in the evolution of bilateral organisms, and encoded for versatile protein scaffolds that could undergo independent sequence diversification to target spectrum. For instance, three potent, broad spectrum AMPs (HFIAP-1, -2, and -3) with cathelicidin structure were isolated from hagfish, a Paleozoic living fossil that appeared on Earth more than 4×10^9 years ago, lacking adaptive immunity and depending essentially on its innate immune system

for survival (Uzzell et al. 2003). In amphibians, a 48-residue amphipathic AMP also with cathelicidin structure was recently cloned from the frog *Amolops loloensis* and functionally characterized as a potent antibacterial and antifungal agent. Molecular phylogenetic relationships established that this amphibian CRAMP predates reptilian but postdates fish cathelicidin (Hao et al. 2012). As most cathelicidins hitherto characterized came from mammals (including humans), this first amphibian cathelicidin filled a gap in the evolution of this AMP family from aquatic to land-living animals. From the venom of Asian elapid snakes, another rather old group of extant land organisms, three CRAMPs were recently reported (Wang et al. 2008; Zhao et al. 2008). Like most members of the vertebrate cathelicidin family, these peptides are biosynthesized as prepropeptide precursors with the first 22 residues corresponding to a signal peptide (leader sequence), followed by a highly conserved proregion (prosequence) comprising the cathelin-like domain and by a hypervariable C-terminal section containing the cationic/amphipathic CRAMP, which is released by proteolysis (Zanetti 2004). These Asian elapid (cobra) peptides were up to now the only examples of reptilian cathelicidins. Some clues about the presence of cathelicidins in the venom glands of the *Crotalinae* (pit viper) subfamily came from a partial gene fragment retrieved from the GenBank EST database from the transcriptome of the venom gland of *Lachesis muta* (Zhao et al. 2008; Junqueira-de-Azevedo et al. 2006). Thus, we wondered if cathelicidin sequences would be expressed in the venom of the most prevalent species of South American pit vipers. The estimated divergence point between pit vipers (*Viperidae* family) and elapid (*Elapidae*) snakes was between 4 and 6 $\times$ 10^8 years ago, *Elapidae* being more modern than *Viperidae* and both families displaying quite distinct venomic profiles, a fact usually related to snake ontogeny, geographical location of habitat, and prey availability (Nawarak et al. 2003; Barlow et al. 2009).

Based on a conserved 5′-untranslated (5′-UTR) region of the cDNA precursors of reptilian cathelicidins, we designed degenerated primers that were used to retrieve vipericidins from pit viper venom gland cDNA libraries. The cDNA precursors were sequenced, translated in silico and their structural characteristics annotated (Table 1). A BLASTx search into a protein non-redundant database returned hundreds of cathelidicin sequences homologous to the cloned ones, with a 32–100 % coverage. Multi-alignment of one hundred such sequences resulted in a cladogram where reptilian cathelicidins were nested close to other sequences from turtles, birds, marsupials, primitive rodents, and ungulate mammals (Figs. 1 and S1). The alignment of the pit viper and the elapid CRAMP sequences (Fig. 2) evidenced a distinguishing signature of *Viperidae* and *Elapidae* cathelicidins (Fig. 3) not present in other vertebrate CRAMPs, namely an additional anionic region between the cathelin and the mature CRAMP domains. This anionic region, of higher variability in both length and sequence than the mature snake CRAMP sequences, is not usual among vertebrates but is found in other non-cathelicidin antimicrobial precursors such as those in the skin of sea (Lu et al. 2008) and fresh water (Wang et al. 2007) amphibians. It has been suggested that the role of this acidic moiety in venom propeptides may be assisting the correct folding of the propeptide by stabilizing the positive charge of the mature sequence, thereby helping peptide storage in secretion granules (Tomasinsig and Zanetti 2005). A further distinguishing feature of these snake venom CRAMPs is the considerable level of sequence conservation in the C-terminal sections, particularly as regards the basic and hydrophobic residues whose eventual α-helical arrangements will confer antimicrobial activity to the CRAMPs encrypted in the cathelicidin precursors (Figs. 2, 3).

After cloning and annotation of the novel CRAMP sequences, expression of their transcripts in the venom glands of the respective pit viper and elapid snakes was confirmed by qPCR. Expression levels differed among the various snake species investigated (Fig. 4), which may be reasonably explained by the ontogenetic peculiarities, i.e., maturity, sex, other individual variables, of the species.

To confirm the antimicrobial activity of the C-terminal sections of South American snake CRAMPs, two vipericidins (crotalicidin and batroxicidin) and Pt_CRAMP1 from the elapid *P. textilis* were synthesized and tested for activity against a panel of standard and clinical Gram-negative and -positive organisms. Given the minimal variations in sequence and physico-chemical properties such as pI and hydrophobicity (Table 2) relative to the other two vipericidins (lachesicidin, lutzicidin) or to Pt_CRAMP1, the three synthesized peptides were judged as a representative of the South American snake CRAMPs in this study. Results (Table 3) confirmed the potent antimicrobial activity of the peptides, particularly of the two vipericidins and to a lesser extent of the elapid-derived Pt_CRAMP1, which underperformed its Asian homolog Oh_CRAMP, also synthesized and tested as a reference. As often the case with other AMPs (Li et al. 2012), vipericidins were significantly more active toward Gram-negative than -positive strains, and showed a decrease of approximately 1-to-2 log units in activity against clinical isolates vs. standard strains. For its part, elapid-derived Pt_CRAMP1 was the weakest antibiotic among all four peptides in this work. The most noticeable structural difference between Pt_CRAMP1 and crotalicidin is the presence of non-polar Met instead of cationic Lys residues at two positions, entailing a rise in hydrophobicity (Table 2) and a corresponding drop in amphipathic helix propensity; the other difference, a Phe/Ile mutation, seems of less consequence. These differences

notwithstanding, the antimicrobial profiles of the CRAMPs tested in this work are altogether impressive, with quite a few MIC values in the low micromolar-to-submicromolar range, hence similar or even better than many conventional antibiotics. In fact, taking into account the MIC values in Table 3, expressed in micromolar concentration, one can note that vipericidins are up to tenfold more efficacious than the control gentamycin (MW 477.596) against both clinical and ATCC isolates (e.g., *P. aeruginosa* and *S. pyogenes*).

Interestingly, the high antimicrobial activity of the two tested vipericidins was complemented by their relatively low cytotoxicity (evaluated as hemolytic activity), particularly for crotalicidin which, even if a bit more toxic than Asian Oh_CRAMP (Fig. 5), still boasts a comfortable 2-log differential selectivity between bacterial and eukaryotic targets. It is also remarkable than the present snake venom peptides are similar to human LL-37 in terms of cytotoxicity (Fig. 5), and less toxic than other reported primate cathelicidins (Zelezetsky et al. 2006; Oren 1999), or than other membranolytic peptides such as mastoparan 7 or melittin (Saar et al. 2005; da Silva et al. 2008). In summary, the excellent bactericidal profile of South American vipericidins highlights these peptides as valuable prototypes for the development of new anti-infective agents.

Since the cathelicidins sequences were obtained from the mRNA expression in the venom glands, it is interesting to know whether these cathelicidins function to protect the venom glands from getting infections or simply act synergistically with other venom components to intoxicate prey. Another point worth considering is the advantage for the snakes to have peptides such as crotalicidin, batroxicidin or Oh_CRAMP that could protect better these glands from microorganisms and be less toxic to the snakes' own tissue than Pt_CRAMP1. More detailed examination of the amino acid sequence of these cathelicidins reveals the greater apparent selectivity—as judged by hemolytic activity—of the vipericidins crotalicidin, and batroxicidin, and also Oh_CRAMP towards prokaryotic cells (bacteria) in comparison with eukaryotic cells (erythrocytes). Further sequence dependence studies and re-engineering of the peptide via amino acid substitutions may provide clues in this direction.

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