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PAPER

Venom peptide analysis of *Vipera ammodytes meridionalis* (Viperinae) and *Bothrops jararacussu* (Crotalinae) demonstrates subfamily-specificity of the peptidome in the family Viperidae

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Snake venom peptidomes are valuable sources of pharmacologically active compounds. We analyzed the peptidic fractions (peptides with molecular masses < 10 000 Da) of venoms of *Vipera ammodytes meridionalis* (Viperinae), the most toxic snake in Europe, and *Bothrops jararacussu* (Crotalinae), an extremely poisonous snake of South America. Liquid chromatography/mass spectrometry (LC/MS), direct infusion electrospray mass spectrometry (ESI-MS) and matrix-assisted desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) were applied to characterize the peptides of both snake venoms. 32 bradykinin-potentiating peptides (BPPs) were identified in the Crotalinae venom and their sequences determined. 3 metalloproteinase inhibitors, 10 BPPs and a Kunitz-type inhibitor were observed in the Viperinae venom peptidome. Variability in the C-terminus of homologous BPPs was observed, which can influence the pharmacological effects. The data obtained so far show a subfamily specificity of the venom peptidome in the Viperidae family: BPPs are the major peptide component of the Crotalinae venom peptidome lacking Kunitz-type inhibitors (with one exception) while the Viperinae venom, in addition to BPPs, can contain peptides of the bovine pancreatic trypsin inhibitor family. We found indications for a post-translational phosphorylation of serine residues in *Bothrops jararacussu* venom BPP (SQGLPPGPPIP), which could be a regulatory mechanism in their interactions with ACE, and might influence the hypotensive effect. Homology between venom BPPs from Viperidae snakes and venom natriuretic peptide precursors from Elapidae snakes suggests a structural similarity between the respective peptides from the peptidomes of both snake families. The results demonstrate that the venoms of both snakes are rich sources of peptides influencing important physiological systems such as blood pressure regulation and hemostasis. The data can be used for pharmacological and medical applications.

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Introduction

Snake venom peptidomes are a very rich but so far mostly unexplored source for drug discovery. Some venom peptides have structural and functional similarities to their human counterparts. They bind to targets influencing vital physiological processes like the hemostasis/blood coagulation and the cardiovascular, and nervous systems. The high target specificity, small size, structural stability and the relative ease of chemical synthesis make the peptides a promising alternative to contemporary drugs. Venom components can be used directly or as prototypes of drugs for the treatment of diseases which do not respond to currently available therapies.[1,2] Some of these compounds have already found preclinical or clinical application for the treatment of hypertension, cardiovascular diseases,

multiple sclerosis, diabetes and pain.[3] A well-known example is the use of the bradykinin-potentiating peptide (BPP), isolated from the *Bothrops jararaca* venom, which served as antetype for the first orally-active inhibitor of the angiotensin-converting enzyme (ACE), named captopril.[4–6] BPPs, natriuretic peptides (NPs) and sarafotoxins (SRTXs) exert profound effects on the cardiovascular system.[7] Snake venom NPs resemble their mammalian counterparts including atrial natriuretic peptides (ANPs), brain natriuretic peptides (BNPs) and C-type natriuretic peptides (CNPs). Mammalian NPs play a crucial role in natriuresis, diuresis and vasorelaxation.[8] A 38-residue peptide (DNP) was isolated from the *Dendroaspis angusticeps* (Green mamba) venom[9] which has been shown to possess vasodilator, natriuretic and diuretic properties, similar to those of the mammalian NPs (ref. 10 and references therein). A synthetic analogue of DNP is today a potent therapeutic agent for the treatment of acutely decompensated congestive heart failure.[11] Snake venom sarafotoxins and mammalian endothelins (ETs) are structurally and pharmacologically related peptides exhibiting a potent vasoconstrictor action. They act on the vascular system *via* identical receptors.[12] Endothelins are very potent vasoconstrictor substances.[13] Disintegrins, found in the venoms of Viperinae and Crotalinae snakes, are small non-enzymatic peptides which selectively block integrin receptors. These receptors are located on the cell surface and mediate cell–cell and cell–matrix interactions.[14,15] Disintegrins and their analogues have the potential to be used as pharmacological tools for the treatment of heart attacks, cancers, osteoporosis and diabetes.[16] A potent peptide antibiotic, cathelicidin-BF, was purified from the venom of *Bungarus fasciatus*.[17]

The examples summarized above illustrate the wide spectrum of pharmacological activities of the snake venom peptides known so far. In the present work we describe and compare the peptidic fractions of two Viperidae snakes from the biodiversity of Europe (*Vipera ammodytes meridionalis*; subfamily Viperinae, Genus Vipera) and South America (*Bothrops jararacussu*, subfamily Crotalinae, Genus Bothrops). *Vipera a. meridionalis* is of public health significance and the most toxic European snake,[18] with unexplored venom peptidome. It is widely distributed in the east part of the continent. *Bothrops jararacussu* inhabits Brazil, Paraguay, Bolivia and Argentina. This snake has an exceptionally large venom output: up to 1000 mg (dry weight) venom can be obtained from a single milking.[19] The venom of *B. jararacussu* is an enormous reservoir of pharmacologically active compounds.

Results

Fractionation of the *Vipera ammodytes meridionalis* venom by size exclusion chromatography and purification of peptides by liquid chromatography

Crude venom of *Vipera ammodytes meridionalis* (25 mg) was fractionated by size exclusion chromatography on a Superdex 75 column at pH 5.0, using 0.1 M ammonium acetate buffer (Fig. 1A). Fractions were collected as shown in Fig. 2. Each peak was subjected to SDS-PAGE under reducing and non-reducing conditions. The first five fractions contained proteins with molecular masses $>$ 10 000 Da (Fig. 2). Fractions 6–11 contained peptides with molecular masses below 10 kDa, which are boxed in Fig. 1A. Peak 6 was further fractionated by liquid chromatography on a Mono S 5 $\times$ 50 column at pH 5.0 (Fig. 3). The first major peak, labelled by arrow, exhibited inhibitory activity towards trypsin. Three peaks were observed after a chromatography of this fraction on a 15 RPC 4.6 $\times$ 100 column (Fig. 4). Electrospray-time of flight mass spectrometry (ESI-TOF-MS) of the first peak showed the presence of two components with masses of 6859 and 7383 Da. The same procedure was applied to the other fractions demonstrating the presence of peptides with masses of 6841 and 7401 Da.

Fractionation of the material from peak 6 is described in detail in order to illustrate the strategy used for isolation and purification of individual peptides from the *Vipera a. meridionalis* venom. The same procedures were applied to the peptide fractions from the other peaks of size exclusion chromatography, and the peptides, isolated, and characterized by ESI-TOF-MS, ESI-FTICR-MS and MALDI-TOF, are listed in Table 1. Peptides with molecular masses of 429–1444 Da were identified in fractions 8–11 (Fig. 1A).

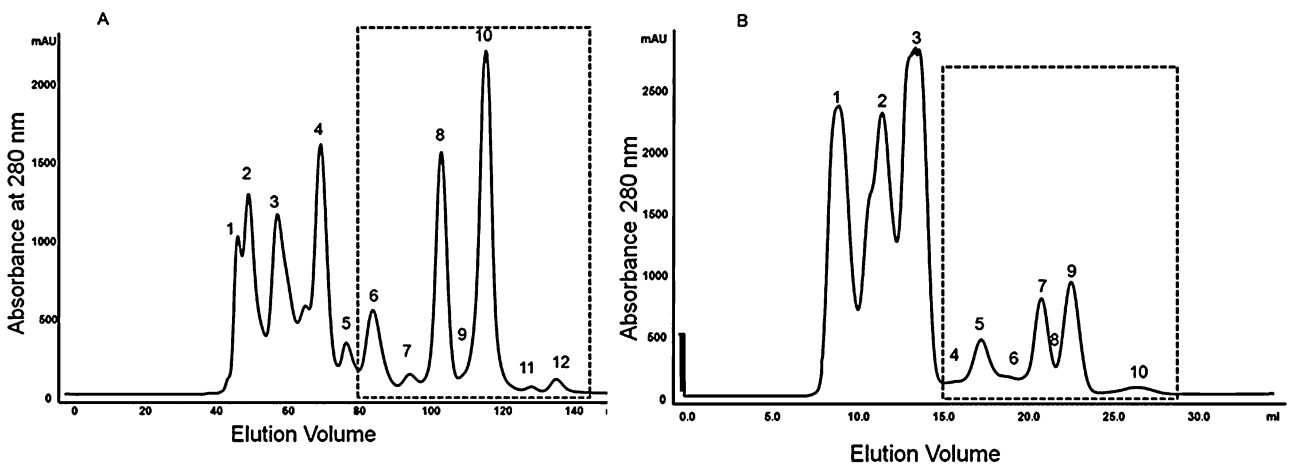

Fig. 1 (A) Size-exclusion chromatography of the *Vipera ammodytes meridionalis* venom on a Superdex-75 column at pH 5.0. The boxed fractions contain peptides below 10 000 Da; (B) size-exclusion chromatography of the *Bothrops jararacussu* venom on a Superdex-75 column at pH 5.0.

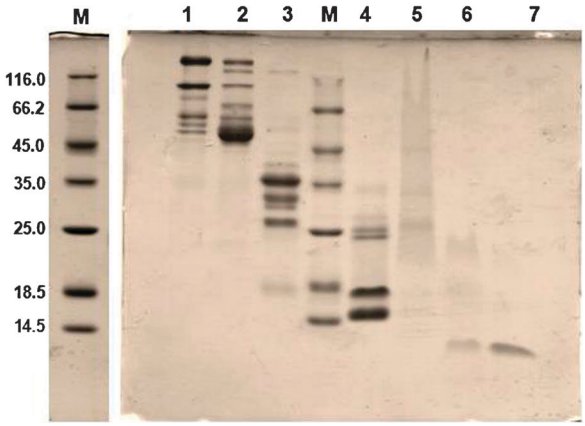

Fig. 2 SDS polyacrylamide gel electrophoresis of fractions 1–7 from the size exclusion chromatographic separation of the *Vipera ammodytes meridionalis* venom. Fractions 1–5 contain proteins with molecular masses above 10 kDa. Fractions 6–7 contain peptides with lower molecular mass.

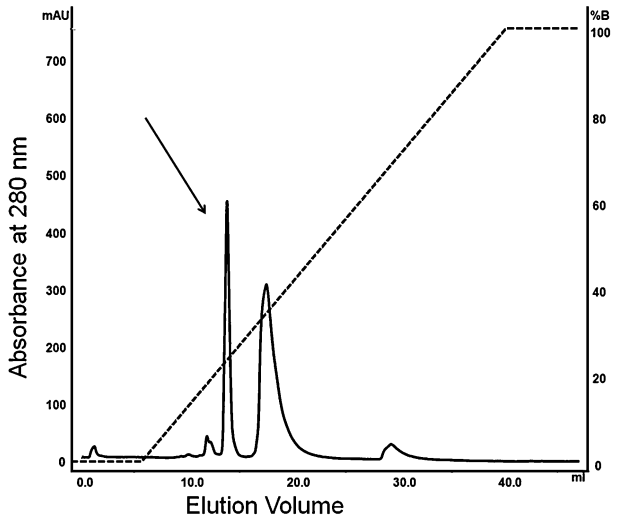

Fig. 3 Fractionation of peak 6, shown in Fig. 1, by FPLC on a Mono S column at pH 5.0.

Fractionation of the *Bothrops jararacussu* venom by size exclusion chromatography and purification of peptides by liquid chromatography

Crude venom of *Bothrops jararacussu* (25 mg) was applied on a Superdex 75 column as described above. The elution profile is shown in Fig. 1B. SDS-PAGE (not shown) demonstrates the presence of proteins with molecular masses > 10 kDa in the first three peaks. Several peaks were observed in the region of low molecular mass peptides (Fig. 1B, peaks 4–10). The fractions from peaks 4–9 were further separated by reverse phase chromatography and the peptides subjected to ESI-TOF-MS or MALDI-TOF-MS analysis. Peptides were identified *via* their m/z value and by MS/MS analysis. m/z values of the observed peptides and their sequences are listed in Table 2.

The peptides, isolated from the venoms of the two snakes, were classified into protein/peptide families using three indexes/properties: molecular mass, enzyme inhibitory activity and amino acid sequence (Tables 1 and 2).

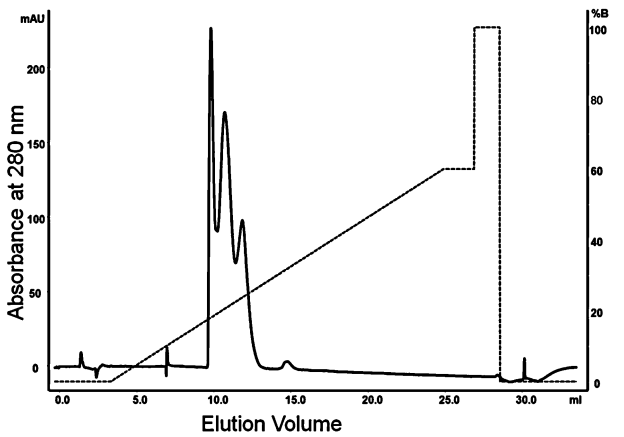

Fig. 4 Chromatography of the first main fraction shown in Fig. 3 on a 15 RPC 4.6 × 100 column. ESI-MS analysis showed the presence of two peptide ions with molecular masses of 6859 and 7383 Da. The second peak contains peptide ions of 6841 and 7383 Da and the third—peptides of 6859 and 7401 Da.

Kunitz type and ACE inhibitors in the *Vipera ammodytes meridionalis* venom

Four signals of a Kunitz type peptide inhibitor (Venom basic protease inhibitor I) were identified in the venom of *Vipera a. meridionalis* (Table 1). The inhibitor has been identified using a tryptic digest analysis by LC/ESI ion trap MS and subsequent data base search. Deconvoluted masses of the intact molecules were M = 6841, 6859.02, 7383.27 and 7401.28 Da. They belong to the 66 amino acid propeptide (M = 7401), the mature inhibitor (M = 6859)[20] and two associated signals of both species with a mass shift of −18 Da which might hint to a pyroglutamate at the N-terminus (mass shift of −17 Da) often reported in snake venom peptides. Further mass differences in both, blocked and unblocked species, point to an amino acid exchange in the *Vipera a. meridionalis* venom peptide compared to its counterpart in the *Vipera a. ammodytes* venom, where this inhibitor has been described, or to post-translational modifications such as amidation.

Four doubly charged ions of m/z 467–591$[M + 2H]^{+2}$ and 12 ions of m/z 430–1445$[M + H]^{+}$ were identified by employing different mass spectrometric techniques, as given in Table 1. Three metalloproteinase inhibitors were sequenced, among which the peptide with a sequence ZRW has been investigated for a first time. The other two peptides ZKW and ZNW have been reported previously in the venom of *Trimeresurus mucrosquamatus*.[21] Z is a pyroglutamyl residue.

Molecular masses between 680–1444 Da are typical for snake venom ACE inhibitors (Table 1). The sequences of seven BPPs were determined as ZPGPVSPQV, ZNWPGPKVPP, PNVTPGCGSVPP, ZRWGPKPVPP, ZNWPGPK and ZRWPGP where Z is a pyroglutamyl residue. Four of these peptides show homology with BPPs and/or with natriuretic peptides from the venoms of Crotalinae (*Gloydius blomhoffii, Bothrops jararaca*, *Sistrurus catenatus* and *Protobothrops mucrosquamatus*) snakes. Moreover, one of the identified

Table 1 Peptide composition of the *Vipera ammodytes meridionalis* venom

Fraction No. (SEC)	Observed *m*/*z*	Sequence determined	Inhibitory activity	Homology with peptide from	Peptide family	Mode of measurement
6	6859.02	QDHPKFCYLPADPGRCA HIPRFYYDSASNKCNKFIY GGCPGNANNFKTWDECR QTCGASA	Trypsin	P00991: *Vipera ammodytes*	Kunitz/ BPTI	Tryptic digestion, LC/ion trap
8	890.60 $(M + H)^+$	ZPGPVSPQV	ACE	P01021.4: *Gloydius blomhoffi*	BPP	Q-TOF, manual sequencing
8	1102.5685 $(M + H)^+$	ZNWPGPKVPP	ACE	B0VXV8: *Sistrurus c. edwardsii*	NP	MALDI-TOF/TOF
8	562.90 $(M + 2H)^{+2}$	PNVTPGCGSVPP	ACE	A8S6B3.1: *Austrelaps superbus*	NP	Q-TOF, sequence determined by PEAKS Online software
8	572.82 $(M + 2H)^{+2}$	ZRWGPKPVPP	ACE	P0C7S7.1: *Protobothrops mucrosquamatus*	BPP	FTICR-MS
8	466.80 $(M + 2H)^{+2}$					
8	1166.50 $(M + H)^+$					
8	1172.50 $(M + H)^+$					
9	681.3019 $(M + H)^+$		ACE		BPP	
9	809.3965 $(M + H)^+$	ZNWPGPK	ACE	B0VXV8: *Sistrurus c. edwardsii*	BPP	MALDI-TOF/TOF
9	570.7646 $(M + 2H)^{+2}$					
9	1159.5913 $(M + H)^+$		ACE		BPP	
10	444.2247 $(M + H)^+$	ZKW		A8YPR6: *Echis ocellatus*	MPI	FTICR-MS
10	723.3574 $(M + H)^+$	ZRWPGP	ACE	Q7T1M3.1: *Bothrops jararacussu,* B0VXV8: *Sistrurus c. edwardsii*	BPP	FTICR-MS
10	1445.7045 $(M + H)^+$		ACE		BPP	
11	430.1721 $(M + H)^+$	ZNW			MPI	Q-TOF, manual sequencing
11	472.2307 $(M + H)^+$	ZRW			MPI	FTICR-MS
11	1276.4733 $(M + H^+)$		ACE		BPP	

Abbreviations used: ACE: angiotensin-converting enzyme; BPP: bradykinin-potentiating peptide; NP: natriuretic peptide; CNP: C-type natriuretic peptide; MPI: metalloproteinase inhibitor; Z means pyroglutamyl residue; SEC: size-exclusion chromatography.

Vipera a. meridionalis peptides showed homology with a natriuretic peptide from the venoms of Elapidae snakes (*Austrelaps superbus*). This indicates a homology between pharmacologically important components of the venom peptidomes of snakes from the two families: Viperidae and Elapidae. Interestingly, the Viper BPPs show an amino acid substitution close to the C-terminal portion of the molecule, when compared to the *Bothrops* peptides. The latter display an isoleucine followed by a double proline, while the former have the isoleucine substituted by valine. Such a feature has also been identified in a *Vipera berus* BPP[22] and might represent a common motif in this genus. The effects of these substitutions on the inhibition of ACE are yet to be investigated.

Structures of the other *Vipera a. meridionalis* venom peptides were not determined due to the low quantities of the isolated material. Search in database (UniProt and Swiss-Prot) showed that they are probably new peptides.

ACE inhibitors in the *Bothrops jararacussu* venom

37 peptides of the peptide fraction of the *Bothrops jararacussu* venom were identified (Table 2). The amino acid sequences of all peptides were determined by MALDI-TOF-TOF. The identified peptides have molecular masses in the range *m*/*z* 700–1883. 32 of them belong to the BPP family of peptide inhibitors involved in the hypotensive effects of the snake venom. The other four are small fragments of snake venom metalloproteinases and of a short neurotoxin (Table 2). In one of the BPPs, with a molecular mass of 1138 Da, having the sequence SQGLPPGPPIP, we found an indication for a phosphorylated serine residue in the *b*-ion series of the MS/MS spectra. However, this result needs further validation. Some of the peptides have previously been reported: ZGRPPGPPIPP, ZNWPHPQIPP, ZARPPHPPIPP, ZARPPHPPIPPAP and ZGGWPRPGPEIPP, where Z means pyroglutamyl residue (ref. 23–26 and references therein). Variability in the C-terminal parts of BPPs was observed. Thus, ZARPPHPP is a modified form of a peptide found in the *B. jararacussu* venom,[24] lacking the C-terminal I-P-P. The peptide ZARPPHPPIPP is another form of ZARPPHPPIPPAP of the *B. jararacussu* peptidome.[24] The peptides QNWPHPQ and ZGGWPRPGP, identified in the present work, are modified forms of peptides from the *B. insularis* and *B. neuwiedi* venoms,[23] lacking the C-terminal I-P-P and E-I-P-P segments, respectively. The structure ZQWAQNWPHPQ is homologous to that of other peptides identified in this work, containing a C-terminal extension of I-P-P (Table 2). ZQWAQNWPHPQIPP is an isoform of a peptide, shown in Table 2, having additional N-terminal Q but lacking a C-terminal I-P-P (Table 2).

Discussion

Comparative analysis of the *Vipera ammodytes meridionalis* (Viperinae) and *Bothrops jararacussu* (Crotalinae) peptidomes demonstrated the presence of representatives of two protein/peptide families: Kunitz/BPTI and BPPs. Our results and literature data point towards a subfamily-specificity of the Viperidae venom peptides. Peptide fractions of the Viperinae snake venom

Table 2 Peptide composition of the *Bothrops jararacussu* venom

Fraction No.	Molecular mass $(M + H)^+$	Sequence determined	Inhibitory activity towards	Homology with peptide from	Peptide family
4	700.3920	RPPHPP	ACE	Q9PW56: *Bothrops jararaca*	BPP and CNP
4	788.4041	ZGRPPGPP	ACE	Q7T1M3: *Bothrops jararacussu*	BPP and CNP
4	882.4584	ZARPPHPP	ACE	Q7T1M3: *Bothrops jararacussu*	BPP and CNP
4	934.5085	SKAPAAPHR		Q9PW56: *Bothrops jararaca*	BPP
4	984.5524	GRPPGPPIPP	ACE	Q7T1M3: *Bothrops jararacussu*	BPP and CNP
4	998.5315	ZGRPPGPPIP	ACE	Q7T1M3: *Bothrops jararacussu*	BPP and CNP
4	999.4832	DLRPDGKQA		A8YPR6: *Echis ocellatus*	SVMPI
4	1007.5560	RPPHPPIPP	ACE	P85167: *Bothrops jararaca*	BPP
				Q7T1M3: *Bothrops jararacussu*	BPP and CNP
4	1078.5571	SGSKAPAAPHR		Q9PW56: *Bothrops jararaca*	BPP
4	1095.5871	ZGRPPGPPIPP	ACE	Q7T1M3: *Bothrops jararacussu*	BPP and CNP
4	1189.6420	ZARPPHPPIPP	ACE	Q7T1M3: *Bothrops jararacussu*	BPP and CNP
4	1139.5407	SQGLPPGPPIP/Phospho (S)		P01021: *Agkistrodon h. blomhoffi*	BPP and CNP
4	1357.7280	ZARPPHPPIPPAP	ACE	Q7T1M3: *Bothrops jararacussu*	BPP and CNP
4	1370.6819	ZGGWPRPGPEIPP	ACE	Q7T1M3: *Bothrops jararacussu*	BPP and CNP
4	1608.8294	ZQWAQGRPPGPPIPP	ACE	Q7T1M3: *Bothrops jararacussu*	BPP and CNP
5	755.4457	ZPPTTKT		P68417: *Naja haje annulifera*	Short neurotoxin
5	791.4226	ZTIGRAY		Q7T1T4: *Bothrops jararacussu*	Zinc metalloproteinase (Fragment)
5	856.4091	ZQWAQGR		Q7T1M3: *Bothrops jararacussu*	BPP and CNP
5	873.4671	ZQKFSPR		Q7T1T4: *Bothrops jararacussu*	Zinc metalloproteinase (Fragment)
5	889.3941	QNWPHPQ		Q7T1M3: *Bothrops jararacussu*	BPP and CNP
5	959.5414	PRPGPEIPP		Q7T1M3: *Bothrops jararacussu*	BPP and CNP
5	1050.5130	ZQWAQGRPP		Q7T1M3: *Bothrops jararacussu*	BPP and CNP
5	1196.5765	ZNWPHPQIPP	ACE	Q7T1M3: *Bothrops jararacussu*	BPP and CNP
5	1386.6845	QGGWPRPGPEIPP	ACE	Q7T1M3: *Bothrops jararacussu*	BPP and CNP
5	1402.6857	QQWPRDPAPIPP	ACE	P86721: *Bothrops atrox*	BPP
5	1883.8993	ZQWAQGGWPRPGPEIPP	ACE	Q7T1M3: *Bothrops jararacussu*	BPP and CNP
6	934.4392	ZGGWPRPGP	ACE	Q7T1M3: *Bothrops jararacussu*	BPP and CNP
6	1384.6777	ZQWPRDPAPIPP	ACE	P86721: *Bothrops atrox*	BPP
6	1709.7891	ZQWAQNWPHPQIPP	ACE	Q7T1M3: *Bothrops jararacussu*	BPP and CNP
				Q6LEM5: *Bothrops jararaca*	BPP and CNP
				Q9PW56: *Bothrops jararaca*	BPP and CNP
				P68515: *Bothrops insularis*	BPP and CNP
7	1581.7651	ZWAQNWPHPQIPP	ACE	Q7T1M3: *Bothrops jararacussu*	BPP and CNP
7	1597.7662	QWAQNWPHPQIPP	ACE	Q7T1M3: *Bothrops jararacussu*	BPP and CNP
7	1597.7662	ZWSQNWPHPQIPP	ACE	B0VXV8: *S. c. edwardsii*	BPP and CNP
7	1613.7647	QWSQNWPHPQIPP	ACE	B0VXV8: *S. c. edwardsii*	
8	1402.6637	ZQWAQNWPHPQ		Q7T1M3: *Bothrops jararacussu*	BPP and CNP
8	1418.4953	QQWAQNWPHPQ		Q7T1M3: *Bothrops jararacussu*	BPP and CNP
				Q6LEM5: *Bothrops jararaca*	BPP and CNP
				P68515: *Bothrops insularis*	BPP and CNP
				Q9PW56: *Bothrops jararaca*	BPP and CNP
9	1293.6139	ZQWAQGGWPRP		Q7T1M3: *Bothrops jararacussu*	BPP and CNP

All the peptides were characterized by MALDI-TOF/TOF. Abbreviations used: ACE: angiotensin-converting enzyme; BPP: bradykinin-potentiating peptide; NP: natriuretic peptide; CNP: C-type natriuretic peptide; MPI: metalloproteinase inhibitor; Z means pyroglutamyl residue; SEC: size-exclusion chromatography.

contain both, a Kunitz type inhibitor and BPPs. The four signals of the Kunitz-type inhibitor were attributed to different peptide species (propeptide, mature peptide and dehydrated peptide with pyroglutamate at the N-terminus) of one peptide inhibitor identical to that isolated from the *Vipera ammodytes ammodytes* venom[20] (Table 1). In the same time, all identified peptides from the venom of the Crotalinae snake, with the exception of several proteolytic fragments of metalloproteinases, belong to the family of bradykinin-potentiating peptides. The published data about the venom peptidome composition of Crotalinae and Viperinae snakes show that in the first case the venom peptide fractions contain BPPs but no Kunitz type inhibitors (with one exception of a very low content, 0.1% and $<0.1\%$ BPTI in the venom of *Sistrurus* rattlesnakes[27]). Thus, BPPs are one of the two major toxic components of the *Bothrops insularis* venom gland (19.7% of the total transcriptome).[28] Bradykinin-potentiating peptides are also among the most frequent transcripts of the *B. jararaca* venom gland (6.2%).[28] High contents of hypotensive peptides were found in the venoms of the other Crotalinae snakes such as *Lachesis muta* (14.7% of the venom proteins),[29] *Lachesis stenophrys* (14.6%),[29] *Bothrops alternatus* (8.8%),[30] *Bothriechis nigroviridis* (26.9%),[31] *Bothriechis lateralis* (11.1%),[32] *Bothriechis schlegelii* (13.4%)[32] and *Crotalus simus* (2%).[33] Small amounts of BPPs (0.8%) were found in the venom of *Bothrops colombiensis*.[34] Kunitz-type inhibitors were identified in the venoms of Viperinae snakes *Vipera ammodytes ammodytes*,[20] *Eristicophis macmahoni* (Leaf-nosed viper),[35] *Bitis gabonica* (Gaboon viper),[36] *Vipera raddei*[37] and *Vipera ursinii renardi*.[38] All these data suggest differences in the evolution of the two Viperidae subfamilies.

The physiological role of the Kunitz type snake venom inhibitors is not clear. They are involved in inactivation of proteases participating in hemostatic processes.[39] It was supposed that these venom peptides participate in the processes of coagulation, fibrinolysis and inflammation through protease inactivation (ref. 40 and references therein). The presence of these inhibitors can also be explained as a strategy to keep venom serine proteases in an inactive state while stored in the gland, thus protecting the snake's tissues. Several Kunitz-type protein inhibitors suppress tumour invasion and metastasis.[41]

In the present work we demonstrate for the first time possible phosphorylation of a serine residue in snake venom BPPs. Protein phosphorylation is a post-translational modification of proteins with the participation of kinases, which is an important regulatory mechanism.[42] The phosphorylation of BPPs results in addition of phosphate groups which would change the peptide electrostatic charge, and might influence BPP–ACE interactions. This can lead to important changes in the hypotensive effect of the bradykinin-potentiating venom peptides.

Table 2 demonstrates a variety of BPPs due to differences in the C-terminal parts. It was shown that the C-terminus is critical for the interaction of BPPs with their natural targets.[43] Changes in this functionally important segment can lead to differences in the pharmacological effects.

BPPs are natural inhibitors of the angiotensin I-converting enzyme (EC 3.4.15.1) that plays a key role in the blood pressure regulation (ref. 44 and references therein). This property makes ACE a pharmacological target for the generation of compounds treating hypertension and cardiovascular diseases. ACE catalyzes the conversion of angiotensin I to the potent vasoconstrictor angiotensin II and the proteolytic degradation of the natural vasodilator bradykinin. BPPs block the formation of angiotensin II and its blood pressure elevating action. In the same time, the breakdown of bradykinin is prevented and its vasodilatory properties help in decreasing the blood pressure.[45] Vasodilatory effects of ACE inhibitors were used by the pharmaceutical industry to produce a large number of antihypertensive drugs such as captopril, enalaprilat, ramiprilat, lisinopril, perindoprilat and other derivatives[44] which are currently used for standard treatment of hypertension and congestive heart failure (ref. 45 and references therein). However, contemporary synthetic ACE inhibitors can induce serious adverse reactions such as renal failure, angioedema, hyperkalemia, urticaria and other undesirable cutaneous effects.[45] Also, a long term therapy with the above-mentioned drugs may result in reduced efficacy of the treatment.[44] A possible reason for the side effects is the relatively low specificity of these drugs or different functions of the angiotensin converting enzyme. ACE contains two independent catalytic domains, the C-terminal being the main site of the angiotensin I hydrolysis.[46] The next generation of ACE inhibitors should act more selectively inhibiting only the N- or C-domain of the enzyme.[44] ACE is a multifunctional enzyme acting on a broad spectrum of substrates as a member of the renin–angiotensin–aldosterone system and non-specific inactivation can induce changes in the function of other physiologically active peptides. In this connection novel, more specific ACE inhibitors will be of pharmacological importance. *Bothrops jararacussu* and *Vipera a. meridionalis* venoms are rich sources of such peptides. The peptide analysis presented here demonstrates the presence of a number of possibly new ACE inhibitors in the venoms of both snakes.

The sequence homology of ACE peptide inhibitors with natriuretic peptides (Tables 1 and 2) suggests processing of larger precursors. It was shown that BPPs are part of two distinct C-type natriuretic peptide (CNP) precursors found in the venom gland and in the brain of another Crotalinae snake, *Bothrops jararaca*.[47] NPs from the *Dendroaspis angusticeps* (green mamba) venom exert vasodilator and natriuretic/diuretic effects.[10] The natriuretic peptide family consists of three subfamilies: atrial NPs and brain NPs which activate the natriuretic peptide receptor A, and C-type NPs activating the natriuretic peptide receptor B.[48] The effects of the NPs are mediated through the activation of the receptors A and B. It can be supposed that at least some of the identified BPPs result from processing of NP precursors. The homology between BPPs and precursors of natriuretic peptides from venoms of Viperidae and Elapidae snakes suggests structural similarities between ACE inhibitors of the first snake family and the respective part of the NP precursors from the venom of the second family.

These peptide homologies in snake genera that are quite distant both from the phylogenetic and geographical point of view are quite remarkable and suggest that these peptides must have appeared at an early stage during the evolutionary process while the fairly high homology rates indicate that a selective pressure must be acting, preserving the BPPs and the natriuretic peptides as vital components of the snake's arsenal.

Experimental

Collection of snake venoms

Vipera ammodytes meridionalis venom was collected from snakes originating from the province Thrace, near the border between Greece and Bulgaria. Crude venom from *Bothrops jararacussu* was obtained from Instituto Butantan (São Paulo; Brazil). Snakes of both sexes were milked. The venom was filtered to remove potential mucosal contaminants, lyophilized and stored at 4 °C until required.

All experiments (the extraction of snake venoms) were performed in compliance with the relevant laws and the guidelines of the Bulgarian Herpetological Society.

Liquid chromatography of crude venoms and peptide fractions

The crude venoms were fractionated by size-exclusion chromatography (SEC). 25 mg of each venom (dry weight) were dissolved in 0.1 M ammonium acetate buffer, pH 5.0, and applied on a Superdex-75 column. The chromatography was performed using the same buffer. UV absorbance of the eluate was monitored at 220 and 280 nm. This step was repeated several times to fractionate about 150 mg of the venom. Fractions were collected and subjected to SDS-PAGE in 15% glycine gel or 18% Tris/Tricine gel under reducing and non-reducing conditions. The gels were stained with Coomassie Blue. Peptide fractions were further purified by liquid chromatography. Separations by HPLC or FPLC were performed on: (a) a Vydac C8 (4.6/150 mm) column with a linear gradient

system of 0–70% consisting of solvent A (0.05% formic acid in H_2O) and solvent B (0.05% formic acid in acetonitrile), to isolate peptides from peaks 8–11, Fig. 1A; (b) on a 15 RPC 4.6 × 100 column with a linear gradient of 0–60% consisting of solvent A (0.05% formic acid) and solvent B (0.05% formic acid in acetonitrile, ACN) to purify peptides from peak 6, Fig. 1A, after Mono-S; (c) PLRP column with an asymmetric gradient of 2–60% consisting of solvent A (20 mM ammonium carbonate) and solvent B (ACN), to further purify the peptides of peaks 4–9, Fig. 1B; (d) Chromolith C18 (4.6/100) column with a linear or asymmetric gradient of 2–40% consisting of solvent A (0.2% formic acid) and ACN as solvent B, to further purify the peptides from the venom of *B. jararacussu*, after the basic RPC. The flow rate was 1 ml min^{-1}.

Liquid chromatography of peptide fraction (peak 6, Fig. 1A) was performed on a Mono S-5 × 50 cation exchange column. Peptides were separated at pH 5.0 with a linear NaCl gradient (0 to 1 M), where solvent A was 0.05 M sodium acetate and solvent B—0.05 M sodium acetate containing 1.0 M NaCl.

Inhibitory activity of snake venom peptides

The inhibition of trypsin by snake venom peptides was analysed using the specific substrate 4-nitroanilide Bz-Phe-Val-Arg-pNA·HCl. The release of 4-nitroaniline upon the enzyme catalyzed hydrolysis of the synthetic substrate was monitored spectrophotometrically at 405 nm. The angiotensin I-converting enzyme activity in the presence of venom peptides was determined by a fluorescence resonance energy transfer assay using Abz-Phe-Arg-Lys(Dnp)-Pro-OH as a substrate.[49] The hydrolysis of the peptide bond between the fluorescent group (*o*-aminobenzoic acid, Abz) and the quencher (2,4-dinitrophenyl group, Dnp) generates fluorescence emission which was followed fluorometrically (λ_{ex} = 320 nm and λ_{em} = 420 nm) at 37 °C.

Tryptic digestion and mass spectrometric identification of larger peptides

Aliquots of the fractions containing peptides of a molecular mass of 6–7 kDa were dried and subsequently dissolved in 6 M urea. To reduce disulfide bridges, 1.3 µl 100 mM dithiothreitol dissolved in digestion buffer (100 mM $NaHCO_3$, pH 8.3) was added and the mixture was incubated at 60 °C for 10 min. Free cysteine residues were blocked with 1.3 µl iodoacetamide (300 mM dissolved in digestion buffer, incubation for 30 min in the dark). 425 µl digestion buffer and 5 µl trypsin solution (sequencing grade modified trypsin; Promega, Madison, USA) at a concentration of 0.25 µg $µl^{-1}$ dissolved in resuspension buffer) were added. The mixture was incubated at 37 °C for 16 h and afterwards the reaction was stopped by adding formic acid at a final pH of 3.0.

Identification was performed on an Agilent 1100 LC/MSD-trap XCT series system. The electrospray ionization system was the Chip Cube system using a Large capacity Chip (Agilent Technologies, Waldbronn, Germany). Sample loading (5–20 µl per sample) onto the enrichment column was performed at a flow rate of 4 µl min^{-1} with the mix of the following two mobile phases at a ratio 98 : 2 (mobile phase A: 0.2% formic acid in H_2O; mobile phase B: 100% ACN). LC gradient was delivered with a flow rate of 400 nl min^{-1}. Tryptic peptides were eluted using a linear gradient of 2–40% B in 40 min. For MS experiments, the following mode and tuning parameters were used: scan range: 300–2000 *m*/*z*; polarity: positive; capacity voltage: 1900 V; flow and temperature of the drying gas were 4 l min^{-1} and 325 °C, respectively. The MS/MS experiments were carried out in auto MS/MS mode using a 4 Da window for precursor ion selection, an absolute threshold of 10 000 after 3 MS/MS spectra. The precursor ion was excluded from fragmentation for one minute. The generic files for database searching were generated by Data Analysis software version 3.4; for precursor ion selection a threshold of 5 S/N was applied and the absolute number of compounds was restricted to 1000 per run. Protein identification was performed with Mascot online search on www.matrixscience.com.[50] MS/MS datasets were used to search the spectra against the subset "other lobe-finned fish and tetrapod clade" of the Swiss-Prot database.[51]

Matrix-assisted desorption/ionization time-of-flight mass spectrometry

MALDI-TOF and MALDI-TOF-TOF analyses were performed on an ultrafleXtreme instrument (Bruker Daltonics, Bremen, Germany). Samples were dried after reversed phase chromatography, resolved in 30% ACN, 0.1% TFA in H_2O and 0.75 µl of the solution was spotted on a MALDI target plate (MTP AnchorChip 384, Bruker Daltonics). After drying 0.75 µl MALDI matrix (0.7 mg ml^{-1} cyano-4-hydroxycinnamic acid (Bruker Daltonics) dissolved in 85% ACN, 1 mM $NH_4H_2PO_4$ and 0.1% TFA dissolved in H_2O) were spotted on the sample spots.

Data acquisition was performed in positive ion mode under the control of the flexControl software 3.3. The parameters were set as follows: ion source 1: 25 kV, ion source 2: 23.6 kV, lens: 7.5 kV. MS data were collected automatically using autoXecute. Parameters were set as follows: laser power: 47%; laser shots: 1000; movement, random walk with 100 shots per raster spot. Peaks were selected for LIFT measurement if they met the following criteria: signal to noise > 8, peak intensity > 300.

MS spectra were processed in flexAnalysis (version 3.3, Bruker Daltonics). Further data analysis was performed using BioTools (version 3.2, Bruker Daltonics) and Mascot Inhouse Search. Mascot[52] version 2.1.03 was used to search the spectra against the subset "other lobe-finned fish and tetrapod clade" of the Swiss-Prot database. The precursor ion mass tolerance was set to 1 Da, the fragment ion mass tolerance was 0.5 Da.

Electrospray ionization time-of-flight mass spectrometry

ESI analysis was performed on TOF LC/MS equipment (Agilent Technologies 6224). The samples were dissolved in 50/50 of 0.1% TFA in ACN and 0.1% TFA in water. 1 µl of the sample was injected at a flow rate of 0.2 ml min^{-1} and an internal standard (ES-TOF reference mix) was used for calibration. Data acquisition was carried out using Agilent MassHunter software (version B.03.01) in positive ESI mode using a gas temperature of 325 °C, a gas flow of 10 l min^{-1}, a capillary voltage of −4000 V and fragmentor voltage was set to 230 V. Data were acquired in a range of *m*/*z* 110 to *m*/*z* 3200.

ESI-QTOF mass spectrometry for peptide sequencing

For protein identification using nanoelectrospray mass spectrometry, experiments were carried out using an electrospray quadrupole time-of-flight mass spectrometer (Q-TOF-2 electrospray mass spectrometer, Waters, Eschborn, Germany) in the positive ion mode. Raw data were acquired and analyzed using the software MassLynx 4.1 (Micromass, Manchester, United Kingdom). Parameters not specified have been the default parameters of the software. The capillary tip voltage was set to 0.70 kV, the cone voltage to 35 V. For CID experiments, ions were selected within a precursor mass window of ±1 Da in the quadrupole analyzer and fragmented in the collision cell using argon as collision gas (Ar) and collision energies of 27 to 35 eV. For peptide identification, peptide tandem mass spectrometry (MS/MS) spectra were deconvoluted by MaxEnt 3 and manually sequenced, supported by PepSeq application for *de novo* sequencing (both part of the MassLinx software package). Peptides with complex fragmentation pattern were sequenced by using the PEAKS Online software, version 5.2

ESI-FTICR mass spectrometry for peptide sequencing

High-resolution mass spectra were acquired using a Finnigan LTQ Fourier transform ion cyclotron resonance (FTICR ULTRA) mass spectrometer (Thermo Fisher, Waltham, USA) equipped with a 7 tesla superconducting magnet. Spectra were acquired at a resolution of 100 000 and the mass error was below 3 ppm at all times. Mass and resolution calibration was performed according to the manufacturer's recommendations. For CID experiments precursor ions were isolated in the linear ion trap using a mass window of 1.5–2 u and were transferred into the FTICR cell after fragmentation. Collision energies were adjusted in order to detect low intensities of the precursor ion (*ca.* 20% relative abundance). Electrospray ionization (ESI) of the samples was carried out using a TriVersa Nanomate (Advion BioSystems). An electrospray voltage of 1.5 kV, a pressure of 0.3 psi and a transfer capillary temperature of 200 °C were applied. Samples were diluted in 0.1% formic acid with a 60% methanol part. For data processing Qual Browser 2.0.7 (Thermo Fisher) was used and the peptide sequencing was performed manually.

Conclusions

Snake venom peptide analysis demonstrates subfamily (Viperinae/Crotalinae)-specificity of the venom peptidome composition in the snake family Viperidae. In contrast to the Crotalinae venoms, the venom peptidome of the Viperinae snakes contains Kunitz type inhibitors. Phosphorylation of a serine residue in BPPs, demonstrated for a first time in the case of *Bothrops jararacussu* venom peptides, can play an important role in the regulation of the BPP–ACE interactions. Also, the venoms of *Bothrops jararacussu* and *Vipera a. meridionalis* are rich sources of novel ACE inhibitors. There is a homology between venom BPPs from a Viperidae snake and venom NPs from Elapidae snakes.

Author contributions

The contributions of the co-authors of the manuscript are as follows: Mrs Aisha Munawar fractionated the snake venoms and isolated individual peptides. Dr Maria Trusch investigated isolated peptides by mass spectrometric methods and determined peptide sequences. Dr Dessislava Georgieva measured enzyme activities of venoms and peptides, arranged the manuscript and participated in the discussion of the results, and in the preparation of the manuscript. Dr Patric Spencer introduced methods for separation of peptide mixtures and for isolation of individual peptides. Dr Violette Frochaux did *de novo* sequencing of some peptides. Dr Sönke Harder did *de novo* sequencing of some peptides. Prof Raghuvir K. Arni delivered the *Bothrops jararacussu* venom and participated in the discussion of the results. Mr Deyan Duhalov selected the *Vipera ammodytes meridionalis* snakes and collected venom used for the investigations. Prof Nicolay Genov discussed and explained results about the snake venom peptidome composition and participated in the preparation of the manuscript. Prof Hartmut Schlüter supervised, discussed and explained the mass spectrometric results. Prof Christian Betzel determined the object of the investigations, organized the experiments, discussed the results and participated in the preparation of the manuscript.

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