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New proline-rich oligopeptides from the venom of African adders: Insights into the hypotensive effect of the venoms

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ABSTRACT

Background: The snakes from the *Bitis* genus are some of the most medically important venomous snakes in sub Saharan Africa, however little is known about the composition and effects of these snake venom peptides. Considering that the victims with *Bitis* genus snakes have exacerbate hypotension and cardiovascular disorders, we investigated here the presence of angiotensin-converting enzyme modulators on four different species of venoms.

Methods: The peptide fractions from *Bitis gabonica gabonica*, *Bitis nasicornis*, *Bitis gabonica rhinoceros* and *Bitis arietans* which showed inhibitory activity on angiotensin-converting enzyme were subjected to mass spectrometry analysis. Eight proline-rich peptides were synthetized and their potencies were evaluated in vitro and in vivo.

Results: The MS analysis resulted in over 150 sequences, out of which 32 are new proline-rich oligopeptides, and eight were selected for syntheses. For some peptides, inhibition assays showed inhibitory potentials of cleavage of angiotensin I ten times greater when compared to bradykinin. In vivo tests showed that all peptides decreased mean arterial pressure, followed by tachycardia in 6 out of 8 of the tests.

Conclusion: We describe here some new and already known proline-rich peptides, also known as bradykinin-potentiating peptides. Four synthetic peptides indicated a preferential inhibition of angiotensin-converting enzyme C-domain. In vivo studies show that the proline-rich oligopeptides are hypotensive molecules.

General significance: Although proline-rich oligopeptides are known molecules, we present here 32 new sequences that are inhibitors of the angiotensin-converting enzyme and consistent with the symptoms of the victims of *Bitis* spp, who display severe hypotension.

1. Introduction

In sub-Saharan Africa, snakebite envenomation is considered a highly relevant public health hazard, of great importance in relation to mortality and morbidity. Although data on incidence and mortality from snakebites in Africa are underestimated, recent reports showed that snakebites represent an important but neglected public health problem among poor rural populations. It has been estimated that a half million snakebites occur every year in Africa, mainly in sub-Saharan Africa, resulting in 100,000 to 500,000 poisonings and 3500 to 32,000 deaths [1].

Snakes belonging to the genus *Bitis*, from the Viperidae family, are implicated in many accidents with humans and are considered medically important vipers. According to the WHO [2], accidents caused by *Bitis arietans* (BA), also known as puff adder, are the most frequent in Africa. Envenomation from these animals results in intense local damage, coagulopathy, thrombocytopenia, spontaneous local bleeding and hypotension [3,4]. Envenomation by *B. arietans* can be extensive and advanced necrosis may require partial or total amputation of the bitten limb, being even fatal in some cases [2]. *Bitis gabonica gabonica* (BG), *Bitis gabonica rhinoceros* (BR) and *Bitis nasicornis* (BN), also known as Gaboon vipers, are responsible for most accidents in southern Nigeria and some of these cases can lead victims to systemic symptoms, such

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as cardiovascular abnormalities like hypotension and shock [5], which in turn can lead to life-threatening envenomation. Despite the high toxicity, fortunately there are not many reported cases of accidents with these snakes in sub-Saharan Africa, and also local effects seen in envenoming cases with Gaboon vipers are less severe than those observed in accidents with BA [2].

Functional evaluations about these venoms demonstrated the presence of peptidases (metallo and serine) [6–8], phospholipases A_2 [9], C-type lectins and hyaluronidase activities [10]. Proteomic analyses showed that metallopeptidases, serine peptidases, disintegrins, L-amino acid oxidase, Kunitz inhibitors, phospholipases A_2, cystatins and C-type lectins are present in all *Bitis* venoms studied [11–13]. Interestingly, only the proteomic analysis of the venom of BG and BR demonstrated the presence of proline-rich oligopeptides (PROs), also known as bradykinin-potentiating peptides (BPPs) [11,12]. Since not all peptides known as BPPs are able to potentiate the effects of bradykinin, although they show inhibition of ACE, they are currently referred to as PROs [14].

The proline-rich oligopeptides present in the *Bothrops jararaca* venom (*Bj*-PROs) were the first naturally occurring angiotensin-converting enzyme (ACE, EC 3.4.15.1) inhibitors described [15]. One of them, the BPP-Va, (<EKWAP, <E = pyroglutamyl residue), is a molecule that originally inspired the design of current commercial inhibitors of ACE [16]. To date, they have also been considered the most potent natural ACE inhibitors and, with few exceptions, they all share the same characteristics, including: 1) a pyroglutamyl residue at the N-terminal; 2) a high content of proline residues and 3) the tripeptide Ile-Pro-Pro at the C-terminus.

The somatic angiotensin-converting enzyme (ACE) is a dipeptidyl carboxypeptidase present on the external surface of endothelial cells. This enzyme, which plays a central role in blood pressure regulation, is composed of two highly similar domains (referred to as the N- and C- domains), each containing an active site, characterized by the presence of a zinc-metallopeptidase HEXXH consensus motif (for review see [17]). In fact, the action of ACE toward circulating vasoactive peptides increases blood pressure by generating angiotensin II (Ang II, Pro-hypertensive octapeptide) and by inactivating bradykinin (BK, hypotensive peptide). The C-domain of mammalian ACE is mainly responsible for Ang II formation while bradykinin is inactivated by both domains with the same efficiency [18]. In fact, some of the proline-rich oligopeptides from snake venoms display selective potency toward the C-domain of ACE [19,20]. Thus, these peptides produce a hypotensive effect in vivo by inhibiting the ACE activity [20], however, other targets that can also induce the cardiovascular effects of different PROs are not exclusively due to the inhibition of ACE [21].

Considering that the victims of accidents with snakes of the genus *Bitis* have exacerbated hypotension and cardiovascular disorders, we investigated the presence of ACE modulators and found a set of new PROs in the fraction of low molecular mass of these venoms, as well as two sequences already described in the venom of *B. jararaca*. The selective ACE domains binded by eight synthetic proline-rich oligopeptides were studied using Ang I and BK as substrates. The in vivo assays were performed to evaluate the physiological effects of these molecules, and to correlate them with the in vitro results.

2. Experimental procedures

2.1. Materials

The lyophilized crude venom of *B. arietans*, *B. gabonica*, *B. g. rhinoceros* and *B. nasicornis* were purchased from Latoxan SAS (Vallence, France). Rabbit lung somatic ACE-I, human angiotensin I and human bradykinin were obtained from Sigma-Aldrich (St Louis, MO, USA). The ACE substrate Abz-FRK(2,4-dinitrophenol)P-OH was kindly provided by Dr. Adriana Carmona (Department of Biophysics, UNIFESP-EPM, São Paulo, Brazil). The synthetic PROs were purchased from GenOne Biotechnologies (Rio de Janeiro, Brazil). The purity of all peptides was analyzed by reverse-phase HPLC and the primary sequences were confirmed by analysis of MS/MS. Acetonitrile acid and trifluoroacetic acid (TFA) were obtained from J.T. Baker.

2.2. Animals

Experiments were performed 43 in adult male Wistar rats weighing 250–280 g. Experimental protocols were approved by the Animal Experimentation Ethics Committee of the Institute of Biomedical Science at the University of São Paulo (ICB/USP). The animals had free access to water and food, and were kept under a 12 h light–dark cycle. All procedures involving animals and their care were conducted in accordance with the Guidelines for the Use of Animals in Biochemical Research.

2.3. Obtainment of the peptide fractions of poisons and HPLC fractionation

Lyophilized venoms of BA (10.6 mg), BG (9.6 mg), BR (9.5 mg) and BN (9.4 mg) were dissolved in 0.05 M ammonium acetate pH 4.2 in a final volume of 5.0 mL, and immediately filtered through a Merck Millipore Amicon Ultracel 10 K centrifugal filter device with a molecular mass cut-off of 10,000 Da (Tullagreen, Carrigtwohill, IRL), in order to prevent proteolytic cleavage of peptides by the crude venom. Filtrated solutions containing low molecular mass fractions were injected (500 μL) in a reverse-phase HPLC (Prominence, Shimadzu, Japan), using 0.1% trifluoroacetic acid (TFA) in water, as solvent A, and acetonitrile and solvent A (9:1) as solvent B. The separations were performed at a flow rate of 1 mL/min using a Restek Ultra C-18 column (4.6 × 150 mm) and a 20–60% gradient of solvent B over 20 min. In all cases, elution was followed by the measurement of ultraviolet absorption (214 nm). The peaks were manually collected, dried and subjected to enzymatic assays.

2.4. Searching for ACE inhibitors

The enzymatic activity assays of ACE were conducted in a 100 mM Tris, 50 mM NaCl, 10 μM $ZnCl_2$ buffer, pH 7.0, using the FRET substrate Abz-FRK(Dnp)P-OH at the concentration of 4 μM, at 37 °C. The reaction was initiated by the addition of ACE (50 ng). The ACE peptidase inhibition assays were performed using 20 μL of each collected peak of the venoms fractionations described above. All reactions were monitored in a continuous assay by measuring hydrolysis using a fluorimeter (Victor 3™, Perkin–Elmer, MA, USA; λem 420 nm and λex 320 nm) and 96 wells Perkin Elmer plates, as described previously by Carvalho and Duzzi et al. [22]. All assays were performed in duplicate, and the specific ACE peptidase activities were expressed as units of free fluorescence of the cleaved substrates per μg of ACE per min (UF/μg/min). The peaks with greater inhibition were sent to MS analysis and de novo sequencing of MS/MS spectra.

2.5. De novo sequencing of peptides by MS/MS

The peptide fractions were automatically injected into a 5 cm C-18 pre-column packed with Jupiter 10 μm resin (Phenomenex; 100 μm I.D.) using the Easy-nLC II system (Thermo Scientific). After the loading process, the peptides were subjected to a chromatographic separation in a 10 cm C-18 column packed with AQUA 5 μm resin (Phenomenex; 75 μm I.D.) at a constant flow rate of 200 nL/min. The peptides were separated with a gradient of 5–15% B (B: 0.1% formic acid in acetonitrile) in 10 min; 15–35% B in 30 min; 35–85% B in 5 min; 85–5% B in 2 min and 5% B in 8 min. The eluate was electro-sprayed at +1.8 kV into an LTQ Orbitrap Velos (Thermo Scientific). The MS spectra were acquired by FTMS analyzer (*scan range*: 400–2000 m/z) with a resolution of 30,000, and the instrument was operated on Data Dependent Acquisition (DDA), where the ten most intense ions per scan were selected for fragmentation by HCD (higher energy collisionally activated

dissociation; 7500 resolution). The minimum threshold for selecting an ion for a fragmentation event was set to 5000 cps. The dynamic exclusion time used was 90 s repeating in intervals of 30 s. The MS and MS/MS spectra were submitted to bioinformatics analyses using the PEAKS Studio 7.0 software (Bioinformatics Solutions Inc.). De novo analysis was performed with tolerances of 15 ppm and 0.025 Da for precursor and fragment ions, respectively, without enzyme specificity. Pyroglutamic acid at N-terminus, Gln or Glu, and methionine oxidation were considered as variable modifications. Peptide sequences were considered with Average Local Confidence scores ≥ 75%.

2.6. In vitro assays

2.6.1. Inhibition of ACE activity by synthetic PROs using FRET substrate

Somatic ACE (10 ng) activity assays were performed in order to determine the inhibition constants (K_i), using 2 concentrations of Abz- FRK(Dnp)P-OH (1 K_m and 2 K_m values, K_m = 4.0 μM) [23] and 5 concentrations of the synthesized PROs (0.4 to 2.0 μM). The fluorimeter and buffer utilized were under the same conditions as described (Section 2.4.). The relative hydrolysis ratio was determined under zero-order kinetics, with <10% of the substrate consumed. Controls without PROs were also performed. K_i values were calculated as described [24]. All assays were performed in triplicate.

2.6.2. Inhibition of ACE activity by synthetic PROs using angiotensin I and bradykinin as substrates

Somatic ACE (50 ng) was incubated in 100 mM Tris, 50 mM NaCl, 10 μM $ZnCl_2$ buffer, pH 7.0, at 37 °C for 60 min with Ang I and BK (30 μM). The concentrations of the substrates used were predetermined to yield measures of maximum velocities (V_{max}) of the reactions (data not shown). The relative hydrolysis ratio was determined under zero-order kinetics, with < 10% of the substrate consumed. Controls without the PROs were also performed. The hydrolysis was analyzed using reverse-phase HPLC (Prominence, Shimadzu, Japan) as described (Section 2.3.). In all cases, elution was followed by the measurement of ultraviolet absorption (214 nm). The peptidase activity inhibition of ACE was determined using 3 μM of each synthetized PRO. The specific activities were expressed in μM of hydrolyzed substrate per μg of ACE per minute (μM/μg/min). All synthetic PROs (10 μM) were tested as possible ACE (50 ng) substrates by incubation period of 4 h–24 h. All assays were performed in duplicate and analyzed with Student's t test.

2.7. In vivo assays

2.7.1. Arterial pressure and heart rate recordings in conscious rats

In order to record mean arterial pressure (MAP) and heart rate (HR), one day before the experiments, the rats were anesthetized with ketamine (80 mg/kg of body weight) combined with xylazine (7 mg/kg of body weight), and a polyethylene tubing (PE-10 connected to a PE-50) was inserted into the abdominal aorta through the femoral artery, while at the same time, a second polyethylene tubing was inserted into the femoral vein for drug administration. Arterial and venous catheters were tunneled subcutaneously and exposed on the back of the rat to allow access to unrestrained freely moving rats. To record pulsatile arterial pressure, MAP and HR, the arterial catheter was connected to a Stathan Gould (P23 Db) pressure transducer coupled to a preamplifier (model ETH-200 Bridge Bio Amplifier), that was connected to a Powerlab computer data acquisition system (Powerlab 16SP, ADInstruments). Recordings were performed 1 day after the surgery and began 30 min after the connection of the arterial line to the pressure transducer. MAP and HR values recorded immediately before, as well as those recorded at the maximum peak of change after i.v. injections of the synthesized peptides, were used as reference to calculate the changes in MAP and HR. Each PRO was tested in one group of animals (n ≥ 4).

2.7.2. MAP and HR in conscious rats that received i.v. PRO

Baseline MAP and HR were recorded for 30 min and then rats received i.v. injections of saline (n = 43). Ten minutes later, rats received i.v. injections of the synthetic peptides (15 μg/kg) and the recordings continued for an additional 30 min. Each PRO was tested in one group of animals (n ≥ 4).

2.8. Statistical analysis for in vivo assay

Statistical analysis was made with Sigma Stat version 3.0 (Jandel Corp., Point Richmond, CA, USA). Data is reported as means ± SEM. Student's paired t test and one- or two-way parametric ANOVA, followed by the Newman–Keuls multiple comparison test, were used as appropriate. Significance was set at $P < 0.05$.

3. Results

3.1. Fractionation of the venoms and searching for ACE inhibitors

After HPLC separation of the low molecular mass constituents (<10 kDa) from all *Bitis* spp venoms, the collected peaks were screened with FRET substrate in order to select the best ones (>90% of ACE inhibition) to proceed with MS analysis. Two fractions from BG, BR, BN and three from BA were sent to MS analysis, as indicated in Fig. 1. Comparative chromatogram profiles (Fig. 1) indicate a correspondence in BG, BR and BN profiles, but a slightly different profile for the BA peptide pool.

3.2. Identification of new peptides from the Bitis venom

De novo sequencing of MS/MS spectra of the selected peaks of *Bitis* venoms resulted in over 150 sequences (supplementary Table S1) with Average Local Confidence (ALC) above 75%, in which almost 20% of them are proline-rich oligopeptides having two noteworthy characteristics, a pyroglutamic acid residue (<E) in the N-terminus, and the fact that most of them present the sequence Ile-Pro-Pro at the C-terminus. Table 1 shows 34 PROs (supplementary spectra S2) containing 6–12 amino acid residues, from four different venoms, whereas the majority are unknown molecules, with exception of <ENWPHPQIPP and <ENWPRPQIPP, respectively named as *Bj*-PRO-10c and *Bj*-PRO-10b, both present in the *B. jararaca* venom [25,26]. Some molecules have a high similarity with described PROs, i.e. <EYWPRP sequence, only differing from BPPVb (<EWPRP, [27]) in the addition of a Tyr after the <E; <ENWPRPK sequence found in BN, matched in 90% with that of *Bj*-PRO-10e from *Bothrops cotiara* [28] and 100% with a bradykinin-potentiating and C-type natriuretic protein (GenBank AFJ49147.1) from *Crotalus adamanteus* [29]. The PRO < EWQRPGPEIPP was the only one found in two species, *B. gabonica* and *B. g. rhinoceros*. Certain PROs sequences presented intraspecific, <ENWPRP*K*IPP and <ENWPRP*Q*IPP (BN), or interspecific, <ENWPHPQ*V*PP and <ENWPHPQ*J*PP (BR and BG, respectively) similarities. In addition, some PROs from BR and BN, including <ENWPRPKIPP and <ENWPRPQIPP, were also found in an extended form composed by a methionine and a lysine residues (MK) right after the double proline in C-terminus (supplementary Table S1).

Differently from the others, *B. arietans* venom presented plenty of sequences with an arginine or a lysine on the C-terminus and only two sequences, <EQRPPRPQIPP and <ERPPRPQIPP, have the Ile-Pro-Pro at the C- terminus.

3.3. Inhibition of ACE by PROs using FRET and physiological substrates

In order to study the activity of *Bitis* PROs in vitro and in vivo, eight of the 34 identified molecules of Table 1 were chemically synthesized. First, the susceptibility of these PROs to ACE hydrolyses was verified on reverse phase HPLC using a C-18 column and, as a result, all peptides remained stable even after overnight incubation (data not shown).

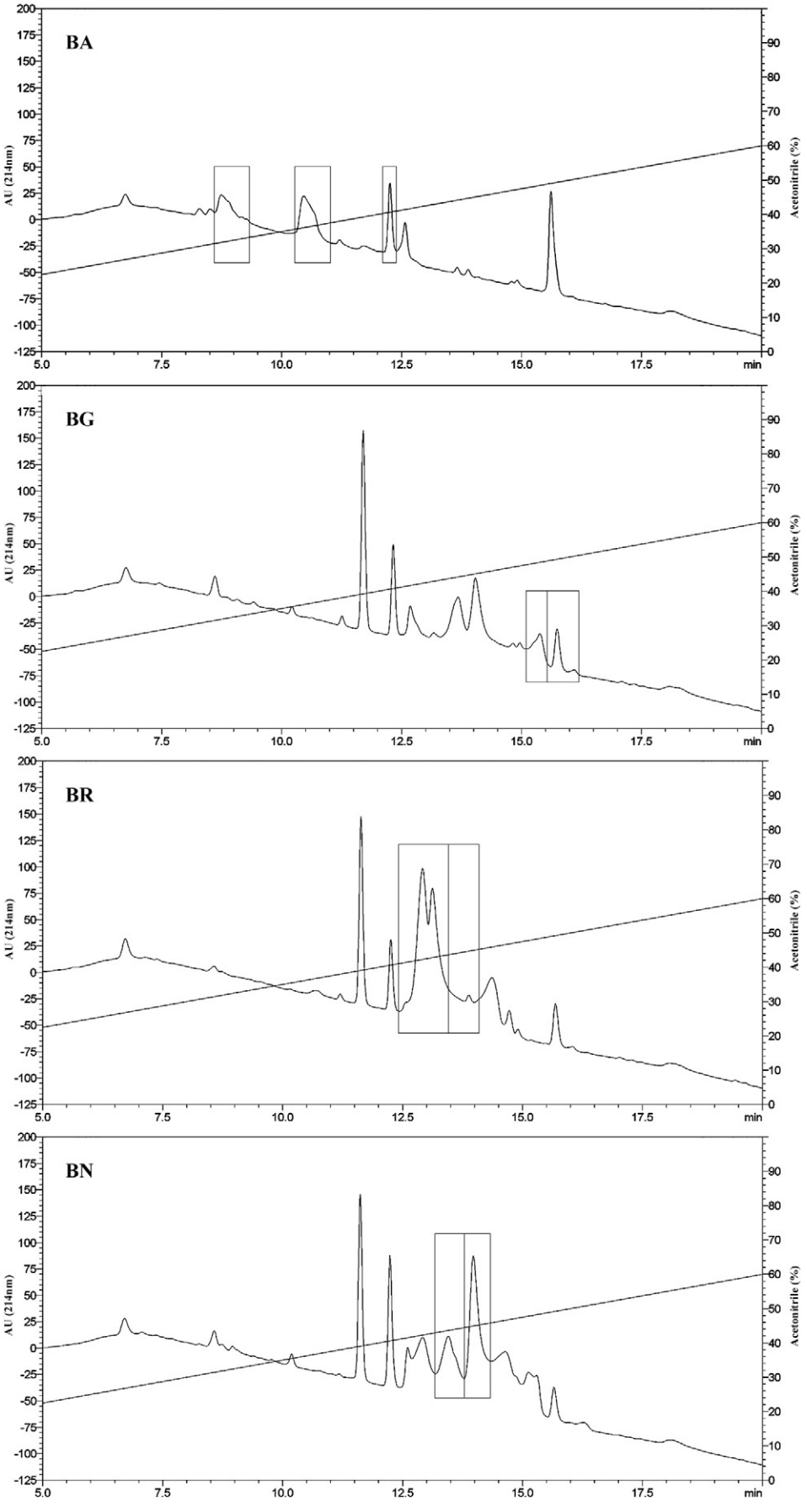
BA
BG
BR
BN
AU (214nm)
Acetonitrile (%)
min
5.0
7.5
10.0
12.5
15.0
17.5

Table 1
Sequence, ALC, mass over charge (m/z) and charges of PROs present in selected fractions from *Bitis* venoms.
The black boxes indicate in which venom the PROs were found.

BA	BG	BR	BN	Sequence	ALC %	m/z	z
		■		<EAMQRGPPEIPP	80	652.3245	2
	■			<EAPKPR	98	340.1982	2
	■			<EAPKPRKQ	87	468.2747	2
■				<EHYPAPK	99	412.2083	2
■				<EHYPAPKK	99	317.8400	3
■				<ELSDPPPK	94	432.7265	2
■				<ELSDPPPR	81	446.7308	2
■				<ELSDPPSRP	80	490.2459	2
	■			<ENWPCCPPIPP	87	412.1816	3
	■			<ENWPHPQIPP[a,b]	81	598.7964	2
		■		<ENWPHPQVPP[a]	81	591.7882	2
		■		<ENWPHPQVPPMK	98	721.3547	2
			■	<ENWPRAPGIPPMK	88	737.8838	2
			■	<ENWPRPGAIPP	85	608.3161	2
			■	<ENWPRPK	99	454.7402	2
			■	<ENWPRPKIPP[a]	82	608.3347	2
			■	<ENWPRPKIPPMK[a]	96	492.2691	3
			■	<ENWPRPQIPP[a,b]	75	608.3175	2
			■	<ENWPRPQIPPMK[a]	93	492.2572	3
			■	<ENWPRQPIPPMK	88	737.8819	2
	■			<ENWQHCEIPP	75	412.1828	3
■				<EQRPPRPQIPP	75	648.8609	2
■				<EQSVPPRP	75	446.2413	2
		■		<ERGPPEIPP	85	487.2583	2
■				<ERPPRP	97	367.2082	2
■				<ERPPRPQIPP	85	584.8328	2
	■			<EWNPHPQIPP	79	598.7962	2
			■	<EWPRPAPGPEIPPMK	99	561.9604	3
		■		<EWQRGPEPIPPMK	78	773.8945	2
		■		<EWQRGPPEIPP	88	644.3274	2
		■		<EWQRPGPEIPMPK	77	516.2654	3
	■	■		<EWQRPGPEIPP[a]	89	644.327	2
			■	<EWRPPAPGPEIPPMK	92	567.2904	3
			■	<EYWPRP	9	415.2029	2

[a] Selected sequences for synthesis.
[b] PROs already described in *B. jararaca* venom.

Next, experiments to determine the inhibition constant of PROs over ACE using Abz-FRK(Dnp)P-OH as substrate on a fluorimeter were performed. As observed in Table 2, the PROs have a K_i range of 0.20 to 0.83 μM, in which the PRO from *B. gabonica* (*Bj*-PRO-10c) presented the best K_i value (0.20 μM), followed by *B. nasicornis* PROs, *Bn*-PRO-10c and *Bj*-PRO-10b (0.25 μM and 0.28 μM respectively). In contrast, peptides with C-terminus –MK extension are poor inhibitors, presenting K_i values of over 100 μM.

Enzymatic inhibition assays using Ang I and BK as substrates were carried out with the aim of investigating the interaction of all eight PROs with C- and N- domains of ACE. Fig. 2 shows that all PROs were able to inhibit ACE activity on both substrates in different degrees. The relative inhibition percentage of BK hydrolysis did not vary significantly among all 8 PROs (19–38%) but, on the other hand, better values of inhibition were obtained when using Ang I as substrate on seven of the eight tested PROs. The exception was for the extended *Bn*-PRO-10b-MK, which inhibits BK hydrolysis of around 19% and Ang I hydrolysis of 11.2%. In fact, as observed in results using the FRET substrate, the two extended peptides, *Bn*-PRO-10a-MK and *Bn*-PRO-10b-MK, have the lowest relative inhibition percentages when using both Ang I and BK as substrates. In addition, *Bn*-PRO-10c, *Bj*-PRO-10b and *Bj*-PRO-10c presented the highest relative inhibition percentage upon Ang I (74.8%, 64.9% and 60.0% respectively).

3.4. Cardiovascular effects of intravenous PROs on conscious rats

All PROs (15 μg/kg) i.v. tested induced an immediate and transitory hypotension (*Bn*-PRO-10a: −18.8 ± 0.3, *Bn*-PRO-10a-MK: −13.9 ± 0.4, *Bj*-PRO-10b: −20.9 ± 1.3, *Bn*-PRO-10b-MK: −20.9 ± 7.0, *Bj*-PRO-10c: −18.7 ± 1.2, *Br*-PRO-10a: −23.3 ± 4.9, *Br*-PRO-11a: −12.6 ± 1.3 and *Bn*-PRO-10c: −18.2 ± 3.1 mmHg, vs. saline: −0.1 ± 0.5 mmHg) as demonstrated on Fig. 3A. Six out of eight PROs also increased HR (*Bn*-PRO-10a: 49.6 ± 1.5, *Bj*-PRO-10b: 37 ± 9.4, *Bj*-PRO-10c: 37.2 ± 5.2, *Br*-PRO-10a: 85.6 ± 10.2, *Br*-PRO-11a: 41.6 ± 13.4 and *Bn*-PRO-10c: 42.5 ± 13.5 bpm, vs. saline: −3.6 ± 1.5 bpm). The only exceptions were *Bn*-PRO-10a-MK (31.8 ± 12.8 bpm) and *Bn*-PRO-10b-MK (3.6 ± 13.7 bpm) which did not change HR (Fig. 3B). The baseline levels of MAP and HR were similar in all eight experimental groups tested, 104.05 ± 3.25 and 366.3 ± 20.47, respectively.

4. Discussion

Snakes of the genus *Bitis* are considered medically important, causing serious envenoming in sub-Saharan Africa. Despite the underreporting of accidents, according to the WHO, the most frequent snakebites are caused by *B. arietans*, while the accidents with Gaboon vipers are less frequent; both cases are associated with serious or life-threatening envenoming [2]. Although there are only a few in-depth studies on envenoming by these snakes, reports of exacerbated hypotension on victims are frequent [3,30,31]. Some studies have associated the hypotension presented by the victims with massive bleeding in the bitten limb [32], but deaths have been reported in patients with circulatory collapse without appreciable blood loss [30]. Thus, a direct action of the venom may be responsible for the hypotension induced in the envenomation by the genus *Bitis*. Given this scenario, we decided to seek molecules present in *Bitis* venoms that could inhibit ACE.

Interestingly, all venoms have pyroglutamyl proline-rich oligopeptides (PROs), containing 6–13 amino acid residues, and at least one proline residue at the C-terminus. Some of the PROs presented here are closely related with sequences already described in the *B. jararaca* venom. Despite the similarities, only two PROs from *B. nasicornis* and *B. gabonica* are identical to the molecules known as *Bj*-PRO-10b and *Bj*-PRO-10c, respectively. It is noteworthy that venoms from snakes of two distant genera would have identical molecules, which has continued the same for about 50 Ma [33].

Some new PROs sequences found in the venoms from Gaboon vipers are extended and show the dipeptide Met-Lys at the C-terminus, e. g., <EWRPPAPGPEIPP*MK*, <EQWQRGPEPIPP*MK*, <EQNWPRQPIPP*MK*, <EQNWPRPKIPP*MK*, <EQNWPHPQVPP*MK*, among others. Previous studies described the isolation of the cDNAs coding for a PRO precursor protein in the venom gland (GenBank AY310916) and in the brain of *B. jararaca* (GenBank AF171670) [20,34]. These proteins contain seven tandemly arranged PROs sequences at the N-terminus, and one C-type natriuretic peptide (CNP) sequence at the C-terminus. Both *B. jararaca* PRO precursor domains comprise three unique copies and two pairs of repeated peptide sequences, all flanked by a highly conserved sequence of seven amino acid residues [L(T/K)VQQWA], except the two pentapeptides <EKWAP [20]. Thus, we suggest that despite the similarity among sequences of PROs of the *B. jararaca* and *Bitis* venoms, precursor molecules probably have striking differences. Extended sequences were not found in the *B. arietans* venom and only two entire PROs, <ERPPRPQIPP and <EQRPPRPQIPP, were detected in the analyzed peaks. Many

Fig. 1. Comparative HPLC profile of low molecular weight constituents from four different *Bitis* species: (BA) *Bitis arietans*; (BG) *Bitis gabonica*; (BR) *Bitis gabonica rhinoceros*; and (BN) *Bitis nasicornis*. Fractions that better inhibited ACE activity was manually collected for MS/MS de novo sequencing, and are framed in each chromatogram. Acetonitrile gradient is represented by the straight line.

Table 2
Inhibition constants (K_i values) of the synthetic PROs for ACE activity using the FRET substrate.

Name	Sequence	K_i (μM)
Bn-PRO-10a	<ENWPRPKIPP	0.48
Bn-PRO-10a-MK	<ENWPRPKIPPMK	>100
Bj-PRO-10b[a]	<ENWPRPQIPP	0.28
Bn-PRO-10b-MK	<ENWPRPQIPPMK	>100
Bj-PRO-10c[a]	<ENWPHPQIPP	0.20
Br-PRO-10a	<ENWPHPQVPP	0.83
Bg-PRO-11a	<EWQRPGPEIPP	0.47
Bn-PRO-10c	<ENWPRPKVPP	0.25

[a] PROs already described in *B. jararaca* venom.

fragments of PROs were detected in the puff adder venom, and since the four venoms studied here were solubilized in the same conditions, we speculate that the *B. arietans* venoms may contain more proteolytic enzymes cleaving the PRO precursor from this venom. Based on the cleavage points, after arginine and lysine residues, it is probable that serine peptidases in *B. arietans* venom are responsible for PROs degradation. Analyzing all PROs together, the set of peptides from Gaboon viper venoms are closely related to each other, while the sequences from puff adder appear to be different. These results are in agreement with studies based on cytochrome b mitochondrial genes [35], NADH subunit 4, 16S and 12S rRNA [33] which grouped the Gaboon vipers in the subgenera *Macrocerastes*, and isolated *B. arietans* in the subgenera *Bitis*.

Among various PROs that were sequenced by mass spectrometry, we decided to synthesize six new sequences along with the two known *Bj*-PRO-10b (<ENWPRPQIPP) and *Bj*-PRO-10c (<ENWPHPQIPP), to check their inhibitory potency on ACE activity using the FRET substrate. The synthetic PROs were not hydrolyzed by ACE, including the two extended sequences (*Bn*-PRO-10a-MK and *Bn*-PRO-10b-MK). Six of them are potent ACE inhibitors, with K_i values between 0.20–0.83 μM, while the two extended sequences show weak ACE inhibitions, even after using higher doses of peptides. As previously stated, *Bj*-PRO-10b and *Bj*-PRO-10c are peptides that are already described for the *B. jararaca* venom, but only *Bj*-PRO-10c has had the inhibition constants determined [19]. Although described nearly 40 years ago [25], only the present report evaluates the *Bj*-PRO-10b, both as ACE inhibitor in vitro and as hypotensive in vivo. The assessment of the relationship between the primary sequence of PROs and the obtained K_i values is not clear. For example, with the intention of investigating whether the IPP tripeptide at the C-terminus is more effective in inhibiting ACE-I than the VPP sequence, it is noteworthy to compare the PROs *Bn*-PRO-10a (<ENWPRPKIPP, $K_i = 0.48$ μM) with the *Bn*-PRO-10c (<ENWPRPKVPP, $K_i = 0.25$ μM), and the *Bj*-PRO-10c (<ENWPHPQIPP, $K_i = 0.20$ μM) with the *Br*-PRO-10a (<ENWPHPQVPP, $K_i = 0.83$ μM). However, no final conclusion has been drawn, indicating that the overall PRO sequence is more important for ACE inhibition than a single amino acid substitution in the primary structure.

The captopril's K_i for ACE was determined as 0.046 μM [23], being over 4 times better when compared with the most potent PRO (*Bj*-PRO-10c). It is important to note that the PROs K_i values were obtained using a FRET substrate specifically developed for the ACE assay, and that the k_{cat}/K_m values for the hydrolysis using two full-length ACE mutants containing only one intact catalytic site (N- or C-domain) were 16.15 $(\mu M.s)^{-1}$ and 25.63 $(\mu M.s)^{-1}$, respectively [23]. Since this substrate is cleaved by both domains, preferably by the ACE's C-domain, we decided to use angiotensin I and bradykinin as substrates to investigate the selectivity of PROs for the ACE C- or N-domain more deeply.

Our HPLC results indicate that four of the PROs sequences *Bj*-PRO-10b, *Bj*-PRO-10c *Br*-PRO-10a and *Bn*-PRO-10c from *Bitis* venoms were better inhibitors for the ACE C-domain since these molecules were better inhibitors for Ang I and for FRET substrate hydrolysis. On the other hand, inhibitions of bradykinin hydrolysis by PROs showed no significant variations, and these four peptides were less effective when compared to results using Ang I as substrate. In accordance with the results obtained with the FRET substrate, the two extended PROs proved to be weaker inhibitors.

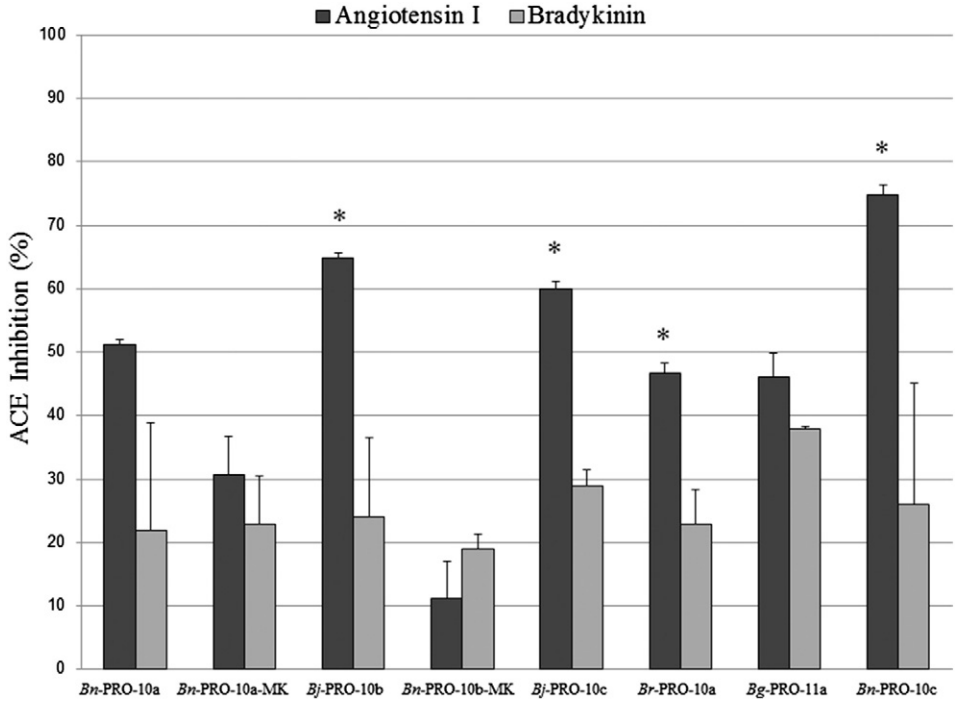

Fig. 2. Inhibition of angiotensin-converting enzyme (ACE) by proline-rich oligopeptides (PROs) from *Bitis* spp over angiotensin I (Ang I) and bradykinin (BK) hydrolysis. Samples of ACE (50 ng) were incubated with 3 μM of each PRO for 60 min when Ang I (30 μM) was used as substrate, and for 120 min with substrate BK (30 μM) at 37 °C. The results are expressed as percentage of ACE activity inhibition and were obtained using a C-18 column at the reverse-phase HPLC system. Experiments were made in duplicate. *Significant difference, using Student's t test $P < 0.05$.

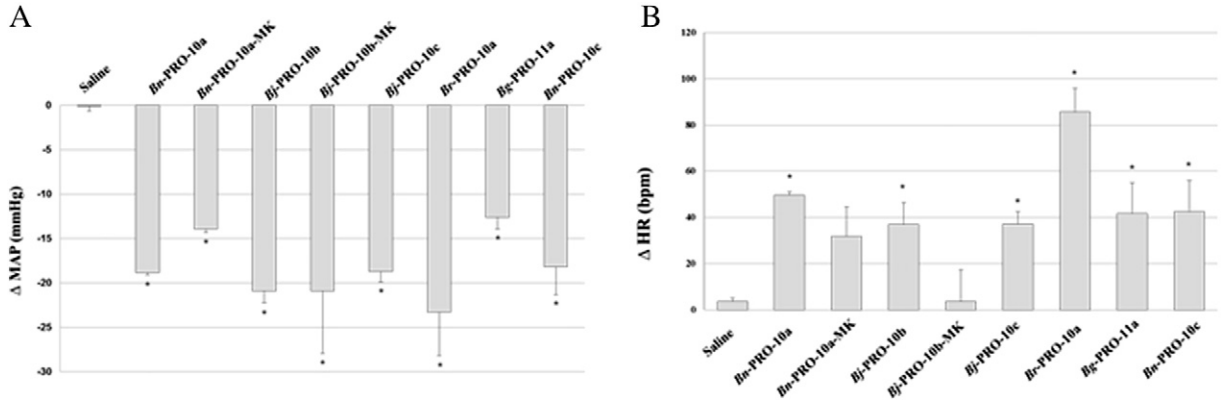

Fig. 3. Changes in (A) mean arterial pressure (MAP) and (B) heart rate (HR) produced by i.v. injection of saline or BPP 1–8 (15 μg/kg). The results are represented as mean ± SEM. N = 4–7 rats/group.

In vivo studies with PROs confirm the hypotensive effect of these molecules, targeting the cardiovascular system of rats. All peptides were able to reduce the mean arterial pressure (MAP) and six of them resulted in tachycardia in the animals.

Although we obtained a good correlation with the in vitro results using different substrates (FRET, Ang I and BK), the same did not occur with the in vivo results. The most significant difference was obtained with both PRO extended sequences, which even though they were not efficient in vitro ACE inhibitors, they resulted in hypotension in vivo, specially the *Bn*-PRO-10b-MK sequence, which was capable of reducing the MAP similarly to other PROs. Interestingly, the extended PROs were the ones that did not produce a significant heart rate (HR) increase.

Taking into account all the results presented here, we hypothesize that the ACE is probably not the only target of PROs in vivo. In fact, it has been shown that *Bj*-PRO-10c has as its main target the argininosuccinate synthetase (AsS) in the kidney cytosol of rats [21], despite being an effective inhibitor of the C-domain of ACE [19]. Another explanation for the good hypotensive effect of the two extended PROs in vivo is that the dipeptide at the C-terminus (MK) may have been removed by other peptidase different from ACE, and that the observed effects are due to the mature PROs.

It is postulated in the literature that a purely C-site selective inhibitor would be beneficial as an antihypertensive drug based on two main aspects: i) it would reduce the vasopressor effect of Ang II by inhibiting its release by the C-domain; and ii) the preserved activity of the N-domain would reduce the BK accumulation that is likely responsible for the side effects of the ACE inhibitors, such as the bradykinin-mediated angioedema [36]. Although both characteristics are present in some PROs, it is noteworthy that these toxins have a relevant role for predation, causing hypotension and facilitating capture of the prey, regardless of the selectivity for specific ACE domains.

The results presented here indicate that a class of bioactive peptides, PROs, most likely contributes to the hypotensive and cardiovascular effects caused by the *Bitis* envenoming. However, we cannot rule out the possibility of other molecules from *Bitis* venoms acting synergistically with the PROs, leading the victims to hypotension. For example, it is kwon that the *B. arietans* venom presents a kallidin-releasing kininogenase from plasma kininogen [8] causing vasodilation, which may be highly potentiated by the ACE inhibitory effect of the PROs. It is important to say that the PROs precursors described so far present natriuretic peptides at the N-terminus [37], which provide another means of achieving hypotension [38]. Since the PROs were selected through a screening using the ACE, it was not possible to identify C-type natriuretic peptides (CNP). However, Francischetti and colleagues [39] described the GLGC sequence (GenBank AY434452) in a transcriptome study with *B. gabonica*, which is highly conserved as one of the four residues of the PROs-CNP precursors.

Taken together, it seems that the venoms of the genus *Bitis* can be considered an arsenal of molecules that leads the victim to hypotensive shock and that clearly require special attention in cases of envenoming.

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

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