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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