Chapter 16

Applications of Snake Venom Proline-Rich Oligopeptides (Bj-PROs) in Disease Conditions Resulting from Deficient Nitric Oxide Production

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1. Introduction

Snake venoms contain a complex mixture of proteins and biologically active peptides [1, 2]. Some of these bioactive peptides are derived from precursor proteins that through proteolytic processing generate mature active polypeptides [3]. As an example, the protein precursor of natriuretic peptide type-C (CNP) from the Brazilian pit viper *Bothrops jararaca* venom and brain originates CNP, a hormone present in several animal species as well as various isoforms of proline-rich oligopeptides (*Bj*-PROs) [4, 5]. *Bj*-PROs were the first natural inhibitors of angiotensin I-converting enzyme (ACE) described [6]. The metalloproteinase ACE, the key enzyme of the renin-angiotensin system, displays two homologous active sites, one at the C-terminal and the other at the N-terminal of the protein [7]. While both active sites convert angiotensin I into angiotensin II and cleave bradykinin (BK) into BK1-5 and BK1-7, the C-terminal is more effective in hydrolysis of these vasoactive peptides [8].

Bj-PROs are molecules of 5 to 14 amino acids residues with a pyroglutamyl residue (<E) at the N-terminus and a proline residue at the C-terminus. *Bj*-PROs longer than seven amino acids share similar features, including a high content of proline residues and a C-terminal tripeptide sequence Ile–Pro–Pro [13]. Since *Bj*-PROs are ACE inhibitors, they potentiate some pharmacological activities of BK, such as induction of contractile action of smooth muscles of guinea-pig ileum *in vitro* as well as *in vivo* BK-induced effects on central nervous, cardiovascular and anti-nociceptive systems [6, 9, 10]. For this reason, these peptides were initially named bradykinin-potentiating peptides (BPPs). The ability of some *Bj*-PROs inhibiting ACE turned them in structural models to develop the first non-peptide site directed inhibitor of this enzyme. The development of Captopril in the early 1980s became a paradigm

for "rational drug design", a concept much heralded today and made possible by computer imaging and genome science [11].

Many studies on structure–activity of *Bj*-PROs showed that a simple analogous structure to Ala–Pro was optimal for binding to the active site of ACE. Replacement of the carboxyl by a sulfhydryl group enhanced the inhibitory activity of the analogue by 1,000-fold. This compound proved to be one of the most potent competitive inhibitors of ACE and, therefore, turned into a useful drug to treat human hypertension (reviewed by [12]). Captopril was a blockbuster drug and inspired the creation of several generations of similar antihypertensive compounds.

However, due to structural diversity of *Bj*-PROs [13] other mechanisms besides inhibiting ACE were proposed. In fact, some of these peptides augmenting argininosuccinate synthase (AS) activity *in vitro* and *in vivo*, can also induce rises in free intracellular calcium concentration ($[Ca^{2+}]_i$) by acting on muscarinic acetylcholine, BK or yet unidentified receptors [15-18] or reversal inhibition of nicotinic acetylcholine receptor [19]. These novel mechanisms of action, recently identified for *Bj*-PROs explain their anti-hypertensive effects [14-17, 20, 21]. Therefore, investigation of *Bj*-PRO-induced effects through acting on different targets opens possibilities of applications for these peptides in the treatment of several pathologies lacking efficient treatment options.

Here, we describe the targets of various *Bj*-PROs and their potential use to treat different target-related pathologies, as well as discuss chemical properties of these peptides for obtaining an oral pharmaceutical formulation.

2. Targets of proline-rich oligopeptides from Bothrops jararaca

Recently, argininosuccinate synthase (AS) was identified as another target for the *Bj*-PROs, which both *in vitro* and *in vivo* positively modulates the activity of this enzyme [14] which leads to L-arginine synthesis [22]. L-arginine is a nonessential amino acid under normal conditions as it is obtained from the breakdown of proteins or synthesized de novo from citrulline in the kidneys by AS (EC 6.3.4.5) and argininosuccinate lyase (ASL, EC 4.3.2.1). AS catalyses the reversible condensation of citrulline with aspartate with consumption of ATP to form argininosuccinate; ASL catalyzes the conversion of the argininosuccinate to fumarate and L-arginine, which is released into the circulation [22].

In the liver, enzymes involved in the anabolism of L-arginine, AS and ASL are present; however, there is not a net production of L-arginine due to arginase activity (EC 3.5.3.1) as part of the urea cycle, catalyzes the hydrolysis of L-arginine into L-ornithine and urea. The urea is then excreted in the urine and L-ornithine is recycled back into the cycle [23]. Furthermore, AS is the rate-limiting enzyme of the citrulline-nitric oxide (NO) cycle for the supply of L-arginine which is then metabolized by NO synthase (NOS) to form NO and citrulline [24-26]. Citrulline, through the reactions catalyzed by AS and ASL may cycle back to arginine, constituting the citrulline-NO cycle [27, 28]. In summary, AS activity contributes to

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three major different functions in the adult organism depending on the cell/tissue considered: (i) ammonia detoxification in the liver, (ii) L-arginine production for the whole organism by kidney and (iii) L-arginine synthesis for NO production in many other cells [22].

Three isoforms of NOS catalyze the reaction: the endothelial constitutive NOS (eNOS), the neuronal constitutive NOS (nNOS) and the inducible NOS (iNOS), reviewed in [29, 30]. NO is a gaseous molecule capable of interacting with many intracellular targets for triggering a series of signal transduction pathways, resulting in a stimulatory and inhibitory signals. NO plays roles in cardiovascular, immune and neuronal control. It is directly involved in arterial tension control since it regulates the local and systemic resistance of vascular walls, as well as the sodium balance [31]. The NO produced by endothelial cells reaches neighboring smooth muscle cells where it activates two types of K^+ channels, ATP-sensitive and Ca^{2+}-dependent [32, 33], and thereby induces the relaxation of blood vessels and brings about vascular dilation leading direct consequences in processes like erection, arterial pressure systemic or organ-specific [34].

Due to the great physiological importance of the NO, compounds revealing properties aspotential NO donors have been protected by patents. They are based on the fact that there are many pathological states related to NO deficiency (reviewed by [35]). However, a major problem of NO donors is to achieve a therapeutic dose without reaching a threshold of toxicity. Mostly, if NO is produced in excess around of cells in a pro-oxidant state, NO could react with reactive oxygen species (ROS) such as superoxide and hydrogen peroxide forming peroxynitrite and nitrogen oxide III, which has been linked to pathogenesis of neurodegenerative disorders [35].

The superoxide anion is produced by uncoupling of NOS due to the lack of its natural substrate L-arginine or tetrahydrobiopterin (BH_4) [36], an important cofactor for NOS. Excessive production of superoxide is explained by increased activity and expression of the enzyme arginase, which competes with eNOS for its substrate L-arginine [37]. Other possible sources for elevated concentrations of ROS include increased expression and activity of NADPH and reduced superoxide dismutase activity [38, 39].

In order to compensate for the deficiency of NO production without induction of toxicity, addition of exogenous L-arginine in the maintenance of NO production has been investigated. The inefficiency of swallowed L-arginine in promoting increase of NO can be explained by its low availability, due to the first pass effect, since the viability of L-arginine as substrate for NOS is reduced by the activity of arginase in the liver. Several studies have shown that induction or activation of arginase may lead to impaired NO production and endothelial dysfunction (reviewed by [35]).

Thus, NO presents challenges and opportunities to intervene and promote human health. The study of regulation of NO production becomes important for understanding the mechanisms which maintain NO levels in a safe range and not injurious to the body.

An important mechanism for control and maintenance of NO levels is achieved by its recycling via the NO-citrulline cycle. The obtained L-arginine provided by the citrulline-NO cycle is then directed to sustain NO production, sparing bulk intracellular L-arginine for other

metabolic roles [24]. In view of that, compounds that increase AS activity and sustain tightly NO production avoiding an excess production will ensure adequate bioavailability for proper physiological functioning. Guerreiro and colleagues demonstrated that a *Bj*-PRO promotes activation of AS, assayed in the presence of the substrates ATP, citrulline, and aspartate, thus leading to NO production by endothelial cells [14]. More recently, we have demonstrated that other *Bj*-PROs induce NO production by activation of AS or kinin-B2 receptors as well as by M1 muscarinic acetylcholine activation, thereby inducing vasodilatation *in vivo* [16, 17].

The patent entitled "Proline-Rich Peptides, Pharmaceutical Composition, use of one or more peptides and method of treatment" was deposited to protect the use and application of *Bj*-PROs and analogous molecules [patent: BR2007/ 000003]. All applications contemplated by patent BR2007/ 000003 are consistent with the use of PROs as prototype molecules for the development of new drugs aiming to treat a range of pathological states related to deficiency in NO production and AS activity, e.g. lung hypertension, preeclampsia, essential hypertension, coagulopathies and citrullinemia. Some of these applications will be discussed below.

3. Pulmonary hypertension

Pulmonary hypertension (PH) is an increase in blood pressure in the pulmonary artery, vein or capillaries, together known as the lung vasculature. In fetus life, PH is a normal state essential for survival. Since the placenta, not the lung, serves as the organ of gas exchange during embryonic development, most of the right ventricular output crosses from the ductus arteriosus to the aorta, and only 5–10% of the combined ventricular output is directed to the pulmonary vascular bed. Pulmonary vascular constriction plays a key role in maintaining high pulmonary vascular tone during fetal life. At the same time, the fetal lung and pulmonary vasculature must prepare for the dramatic adaptation to air breathing at the time of birth [40].

However, PH can continue even after the birth, called persistent pulmonary hypertension that develops when pulmonary vascular resistance remains elevated, resulting in right-to-left shunting of blood through fetal circulatory pathways. The pulmonary vascular resistance may remain elevated due to pulmonary hypoplasia and cause disease states, like congenital diaphragmatic hernia, as well as impaired development of the pulmonary arteries, resulting in the meconium aspiration syndrome, or failing adaption of the pulmonary vascular bed as occurs with perinatal asphyxia [41]. Moreover, PH has more than one etiological factor, thus it can be classified as idiopathic PH when there is no identifiable cause of this disease; familial PH with a previous disease history; and associated PH when an underlying cause of PH such as connective tissue disease is present [42].

PH has been reported in patients with chronic hemolytic anemias, including sickle cell disease, thalassemia, paroxysmal nocturnal hemoglobinuria, hereditary spherocytosis, malaria, among other disease states [43, 44]. The exact mechanism(s) involved in the development of

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PH in these patients is unclear. Hemolysis may result in a nitric oxide deficient state through free hemoglobin scavenging of nitric oxide and release of erythrocyte arginase, which limits L-arginine, a substrate for nitric oxide synthesis [45].

Nitric oxide is synthesized from terminal nitrogen of L-arginine by NOS. All three NOS isoforms are expressed in the lung and are distinguished by regulation of their activities, as well as by specific sites and developmental patterns of expression [46]. The isoform eNOS is expressed in vascular endothelial cells and is believed to be the predominant source of NO production in pulmonary circulation [40]. This hypothesis is corroborated by the fact that NO inhalation in premature newborns with severe respiratory failure due to PH provides improvement of symptoms, accompanied by marked increase in oxygenation [34].

Although large well-designed studies paved the way to Food and Drug Administration (FDA) approval of therapeutic NO inhalation, it is equally important to note that inhaled NO did not reduce the mortality, length of hospitalization, or the risk of significant neurodevelopmental impairment associated with persistent PH in newborn children [40]. It is known that at excessive levels NO can react with reactive oxygen species (ROS) such as superoxide and hydrogen peroxide. Such increase in ROS was observed in the smooth muscle and adventitia of pulmonary arteries from lambs with chronic intrauterine PH [47, 48], forming peroxynitrite, an anion with deleterious tissue-oxidant effects [49].

Inhaled NO is usually delivered with high concentrations of oxygen. Whereas hyperoxic ventilation continues to be a mainstay in the treatment of PH, little is known about the side effects of oxygen supply together with NO. The extreme hyperoxia routinely used in PH management may in fact be toxic to the developing lung due to ROS formation [39, 50, 51]. Superoxide may react with arachidonic acid to increase concentrations of isoprostanes and may also combine with NO to form peroxynitrite [52] with possible induction of vasoconstriction, cytotoxicity, and damage to surfactant proteins and lipids. Moreover, peroxynitrite has been shown to directly induce NOS uncoupling. New data indicate that even brief (30 min) periods of exposure to 100% O_2 are sufficient to increase reactivity of pulmonary vessels in healthy lambs [53, 54], to diminish the response of the pulmonary vasculature to endogenous and exogenous nitric oxide [54], and to increase the activity of cGMP-specific phosphodiesterases [51]. Inhaled NO would theoretically benefit patients with chronic primary or secondary pulmonary hypertension, but its therapeutic application in this setting has been limited by the risk of causing rebound pulmonary hypertension, if it is inadvertently discontinued, and the lack of practical home-based continuous delivery devices.

Therefore, we have proposed an alternative approach for controlled induction of NO production. We believe that cytosolic L-arginine provides a major NO donor. Arginine concentrations subject to metabolic fine-tuning controls will assure that the amino acid is kept in a homeostatic concentration range. These effects could be achieved by the action of *Bj*-PRO on AS, a target unexplored by the pharmaceutical industry. Compounds inducing an increase in AS expression and activity are promising for the treatment of diseases related with deficient NO production.

4. Preeclampsia

Preeclampsia, a pregnancy-specific syndrome characterized by hypertension, proteinuria and edema, causes fetal and maternal morbidity and mortality with high incidence in developing countries [55]. Symptoms of preeclampsia are currently combated by sodium restriction, rest and medication for blood pressure control to avoid complications for the mother and prolong the pregnancy for fetal maturation [56-58]. However, this attempt is rather unspecific with possible side effects for the developing fetus [59, 60]. Currently, the only therapy of preeclamsia involves placenta removal resulting in pre-term birth [61]. Therefore, novel drug development for pregnancy-specific conditions remains a challenge [59].

The pathology of preeclampsia involves systemic inflammation, oxidative stress, alterations in the levels of angiogenic factors, and vascular reactivity leading to hypertension of the mother and metabolic alterations in the fetus [61, 62]. A number of evidence suggests that clinical manifestations are caused by endothelial malfunction including insufficient production of NO [63, 64]. Levels of eNOS, the enzyme responsible for NO synthesis in the endothelium from L-arginine, are decreased in human umbilical vein endothelial cells from pregnant women suffering from preeclampsia [62] together with impaired AS expression [65]. The low availability of L-arginine uncouples eNOS activity, decreases NO production and increases eNOS-dependent superoxide generation [62, 66], consequently resulting in reduced vasodilatation or in inflammatory processes observed in preeclampsia [66, 67]. Therefore, it is expected that the sustained concentration of L-arginine in endothelial cells is likely to play a critical role not only in the control of systemic blood pressure, but also in inhibition of inflammatory processes [68].

Recently, we have reported that a *Bj*-PRO containing ten amino acid residues (*Bj*-PRO-10c), activating AS, is able to correct dysfunction of human umbilical vein endothelial cells from pregnant women suffering from preeclampsia [65] (Figure 1).

Bj-PRO-10c, besides augmenting the activity of AS both *in vitro* and *in vivo* [14] increases significantly eNOS expression of human umbilical vein endothelial cells obtained from pregnant women suffering from preeclampsia [65]. It was observed that the increase in NO levels induced by *Bj*-PRO-10c diminished the oxidative stress of the endothelial cells of preeclamptic women, shown as a 50% reduction in superoxide levels [65] (Figure 1).

Most importantly, *Bj*-PRO-10c promoted NO production only in endothelial cells from patients suffering from the disorder and not in normotensive pregnant women. In agreement, *Bj*-PRO-10c is a molecule endowed with antihypertensive activity that reduced blood pressure in hypertensive but not in normotensive rats [69]. These observations led to suggest that *Bj*-PRO-10c promotes its anti-hypertensive effect in mothers with preeclampsia without any effect on the blood pressure of the fetus, a problem with drugs currently used for minimizing health problems arising from preeclampsia. Taken together, *Bj*-PRO-10c becomes a potential tool for the development of an efficient drug for preeclampsia treatment.

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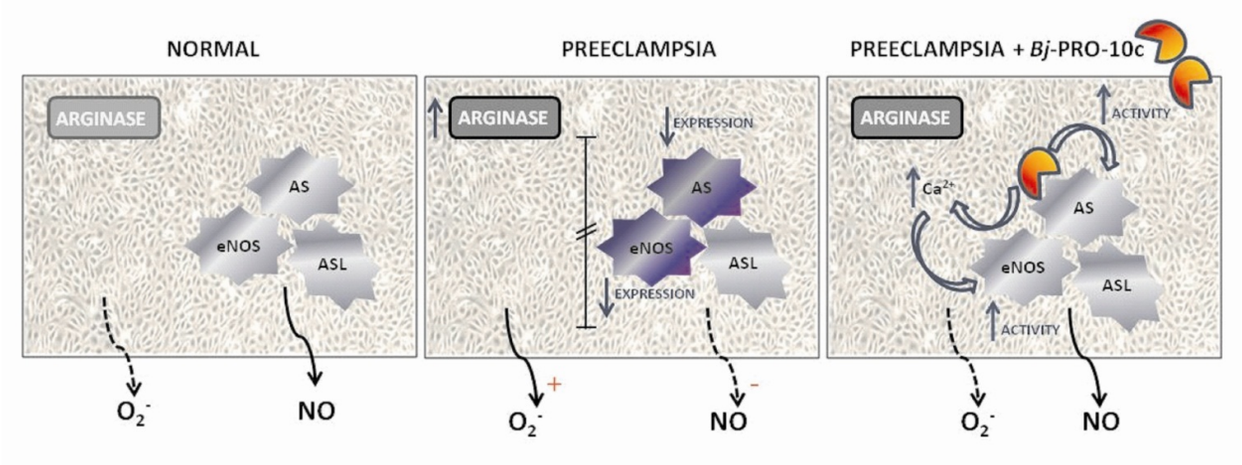

Figure 1. *Bj*-PRO-10c-induced effects on endothelial cell from healthy and pregnant women suffering from preeclampsia. In endothelial cells from normotensive pregnant women (NORMAL), NO production is adequate to maintain normal gestation. In cells from patients with the disease (PREECLAMPSIA) both decreased NO production and increased superoxide (O_2^-) production are observed together with enhanced activity of the enzyme arginase, reduced expression of eNOS and AS and uncoupled eNOS. When endothelial cells from pregnant women suffering from preeclampsia are exposed to *Bj*-PRO-10c (PREECLAMPSIA + *Bj*-PRO-10c), production of NO and superoxide return to normal ranges, since *Bj*-PRO-10c initiates a signaling cascade including increases of cytosolic calcium concentration activating eNOS and augmenting AS activity.

5. Citrullinemia

Citrullinemia, a disorder causing serious episodes of neurological symptoms associated with hyperammonemia involving disorientation, abnormal behaviors (aggression, irritability, and hyperactivity), seizures, coma, and potentially death from brain edema [70], occurs in two variants: CTLN1 (MIM#215700) or classical neonatal onset, and CTLN2 (MIM#603471) or adult-onset [71-74]. Classical citrullinemia in children is associated with a mutation in the AS gene [75]. However, in CTLN2 the enzyme reveals normal kinetic properties and is quantitatively deficient only in the liver of adult-onset citrullinemia patients [71-74]. The most successful therapy of CTLN2 has been liver transplantation [76, 77] because this treatment prevents hyperammonemic crises and corrects consequent metabolic disturbances [70].

It has been reported that administration of L-arginine to CTLN2 patients is effective in decreasing blood ammonia concentration [78, 79]. For ammonia detoxification, arginine needs to enter the liver via the portal vein where is metabolized by mitochondrial arginase to provide ornithine for citrulline and aspartate synthesis and for the priming of the urea cycle [80]. However, care must be taken when administering L-arginine, as fatal cases caused by L-arginine hydrochloride overdose have been reported [81]. In general, the dose of L-arginine supplementation used in the treatment of hyperammoaemia is in the high range between 100 and 700 mg/kg body weight per day [82-85]. In animal models the effects of hyperargininaemia can be observed, reflecting toxic effects of high L-arginine concentration

and making it possible to predict side effects of L-arginine supplementation including cognitive deficits, epilepsy and a progressive spastic diplegia [86]. Therefore, drugs augmenting AS activity, the step-limiting enzyme of urea cycle, may be a promising strategy for CTLN2 therapy, since AS is the step-limiting enzyme of the urea cycle. A consequence of increased AS activity, a final common pathway is triggered resulting in the excretion of waste nitrogen as urea [87].

As previously mentioned, CTLN2 is not associated with genetic mutation of the AS gene; however, Saheki et al. identified the SLC25A13 gene as being defective in CTLN2 patients. This gene encodes for a Ca^{2+}-dependent mitochondrial solute carrier, designated citrin [88]. According to Saheki et al., it is difficult to predict disease-causing effects of citrin deficiency in CTLN2, since children carrying citrin gene mutations may suffer from CTLN2 after more than 10 years or several decades of being asymptomatic [70]. In view of that, an option to prevent CLTN2 in infants with mutation of the citrin gen is being sought, having in mind that the nutritional management with appropriate intake of proteins only avoids accumulation of nitrogen [70].

Based on information discussed here, the strategy to increase AS activity in the liver could be an effective treatment for CTLN2 as well to prevent that children diagnosed as carrying SLC25A13 mutations from developing CTLN2 in the future [89]. We believed that direct pharmacological and clinical studies with *Bj*-PROs for these proposals, could turn them into a powerful therapeutic tool. The efficiency of *Bj*-PRO action can yet be improved by the rational design of a compound which in the liver accelerates the urea cycle for eliminating ammonia or even preventing its accumulation.

6. Conclusion

AS as molecular target for drug development will be important for the treatment of a wide variety of diseases associated with deficiency of NO production, and also could transform *Bj*-PROs or their synthetic analogous into blockbuster medicine, as happened in the 80s with Captopril [90, 91]. The properties of *Bj*-PROs enhancing AS activity [14, 17], provides a precise pharmacological tool for controlling pathophysiological mechanisms with advantages of uncontrolled application of exogenous L-arginine or NO donors [92]. For instance, the effect of exogenous NO donors is not subject to physiological control, thus being more susceptible of generating undesired ROS [93, 94]. For all these reasons, keeping NO production in a safe level, so that a deleterious threshold would not be reached, is of particular interest. In this way, *Bj*-PROs should serve as structural models for the development of therapeutic agents for the treatment of various diseases related to NO deficiency, as cause or effect, as well AS deficiency.

Chemical properties of *Bj*-PROs make these peptides even more attractive potential lead compounds for drug development. For instance, *Bj*-PRO-10c is able to penetrate cells, where it remains as an intact molecule for hours [14]. Moreover, *Bj*-PROs contain a notable high proline content [13], which gives them some resistance to hydrolysis by aminopeptidases,

carboxypeptidases and endopeptidases [95]. Nevertheless, cyclodextrins or nanocompounds could provide carriers for *Bj*-PROs, since drug release could be controlled in accordance with therapeutic propose [96, 97].

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