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Review

Bradykinin-potentiating peptides: Beyond captopril

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ABSTRACT

The identification of novel endogenous and exogenous molecules acting in the complex mechanism of regulating the vascular tonus has always been of great interest. The discovery of bradykinin (1949) and the bradykinin-potentiating peptides (1965) had a pivotal influence in the field, respectively, in understanding cardiovascular pathophysiology and in the development of captopril, the first active-site directed inhibitor of angiotensin-converting enzyme, and used worldwide to treat human hypertension. Both discoveries originated from studies of envenoming by the snake *Bothrops jararaca*. The aim of the present article is to reveal that the snake proline-rich oligopeptides, known as bradykinin-potentiating peptides, are still a source of surprising scientific discoveries, some of them useful not only to reveal potential new targets but also to introduce prospective lead molecules for drug development. In particular, we emphasize argininosuccinate synthetase as a new functional target for one of bradykinin-potentiating peptides found in *B. jararaca*, *Bj*-BPP-10c. This decapeptide leads to argininosuccinate synthetase activation, consequently sustaining increased nitric oxide production, a critical endogenous molecule to reduce the arterial blood pressure.

1. Introduction

Before the advent of high throughput structure-based drug design, the drug discovery process relied on lengthy and unglamorous work on the effect natural products could have on cell/organ/whole animal physiology. This traditional approach is vanishing since the 1990s under a massive expansion of synthetic medicinal chemistry (Li and Vederas, 2009). Yet, for details on the mechanisms of action and determination of binding sites at the structural level, it is unlikely that rapid screening methods will be suitable, as there are requirements for detailed, tailor-made experimental design and data interpretation, which are rarely in accordance with the concepts of mass high throughput screening. Although scarcely used nowadays, the expensive, low throughput classical method for the development of animal toxin-derived drugs is still opening new and exciting opportunities, as will be reviewed in this article. Here we describe how snake venom toxins provided avenues for the discovery of new, yet unsuspected targets which may be useful to design novel biopharmaceuticals to treat human cardiovascular dysfunctions, particularly human hypertension.

2. The angiotensin-converting enzyme (ACE) inhibitors

The impressive advances on the ACE inhibitors originated in 1949, when Mauricio Rocha e Silva et al. discovered bradykinin (Bk), while studying the pathophysiology of *Bothrops jararaca* (*Bj*) envenoming (Rocha e Silva et al., 1949). This discovery, which greatly enhanced the comprehension of blood circulation, motivated Brazilian scientists and others all over the world to study the role of Bk in cardiovascular physiology and pathology. This

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allowed the uncovering of the complex multistep reactions of the kallikrein-kinin system, whose activation by *Bj*-serine proteases leads to the generation of the hypotensive peptide bradykinin from plasma kininogen (Schmaier, 2002; Serrano et al., 1998). It has taken over three decades to understand the contribution of the kallikrein-kinin system to the physiology of blood circulation (Linz et al., 1995). The kallikrein-kinin system regulates the physiological level of Bk and other kinins. Kinins are implicated in various physiological processes, including the regulation of blood pressure. This complex system includes the serine proteases tissue- and plasma-kallikrein, which release kinins from kininogen. Bradykinin exerts its pharmacological activities by binding to specific receptors, before being metabolized mainly by somatic ACE (sACE) (Moreau et al., 2005) (Fig. 1). In this context, one important conclusion was that the activation of the kallikrein-kinin system by the snake venom would lead to hypotensive shock if it was not counterbalanced by physiological antagonist systems, including the renin-angiotensin system, identified a few decades earlier (Tigerstedt and Bergman, 1898; Goldblatt et al., 1934).

Similarly to the kallikrein-kinin system, activation of the renin-angiotensin system is a multistep proteolytic event that generates angiotensin I (Ang I), the immediate precursor of the hypertensive peptide angiotensin II (Ang II) (Skeggs et al., 1981). Ang II is an octapeptide that results from the hydrolysis of the C-terminal dipeptide of Ang I, catalyzed by sACE (Fig. 1). Ang II in turn may be converted to angiotensin-(1-7), a vasodilator, by ACE2 (Tipnis et al., 2000; Vickers et al., 2002), and angiotensin-(1-7) can be further inactivated by sACE (Santos et al., 2008). Thus, both the renin-angiotensin and kinin-kallikrein systems contribute to cardiovascular homeostasis (Fig. 1).

Interestingly, the activity of a single enzyme, sACE, strategically located at the plasma membrane of endothelial cells, is essential as it regulates the arterial blood pressure by generating Ang II and inactivating Bk (Acharya et al., 2003). In fact, after the hydrolysis of plasma angiotensinogen by aspartyl protease renin, Ang I (inactive) is converted into the potent vasopressor peptide, Ang II, and the arterial blood pressure goes up. The importance of sACE for blood pressure regulation was recognized by Ng, Ferreira et al. who suggested sACE as a putative target to develop drugs (Ng and Vane, 1967, 1970; Ferreira et al., 1970a). As a consultant of The Squibb Institute for Medical Research, Vane motivated two young scientists, David Cushman and Miguel Ondetti, to search for inhibitors of sACE action. They ultimately developed captopril, a potent inhibitor of sACE (Cushman et al., 1977).

A preliminary step toward the development of captopril was taken by Ferreira and Rocha e Silva during the search for inhibitors of Bk degradation. They suspected that the major enzyme for Bk degradation in blood vessels was a metallopeptidase, thus, they used BAL (dimercaptopropanol) to chelate the metal ion, the enzyme cofactor, to inactivate the enzyme. In fact, BAL enhanced the smooth-muscle contraction elicited by Bk several fold. This work established for the first time a clear connection between Bk potentiation and degradation (Ferreira and Rocha e Silva, 1962). Interestingly, almost simultaneously eight inhibitors of kinin inactivation were reported, six of which were shown to have SH groups (Erdös and Wohler, 1963). Moreover, Ferreira and Rocha e Silva also found out that the *Bj*-venom contained peptides that greatly enhanced the smooth-muscle contracting activity of Bk, most likely by a similar mechanism attributed to BAL, i.e., by inhibiting Bk degradation (Ferreira and Rocha e Silva, 1965). One of these peptides was the pentapeptide *Bj*-BPP-5a (<Glu–Lys–Trp–Ala–Pro), the first *Bj*-BPP to have its amino acid sequence determined (Ferreira et al., 1970b). Later, it was shown that these bradykinin-potentiating peptides from *Bj*-venom (*Bj*-BPPs) belong to a family of snake venom proline-rich oligopeptides whose biological properties are associated with impairment of Ang II generation and Bk degradation (Hayashi and Camargo, 2005; Menin et al., 2008).

The evidence that sACE inhibition could be useful for the treatment of human hypertension came from the work of

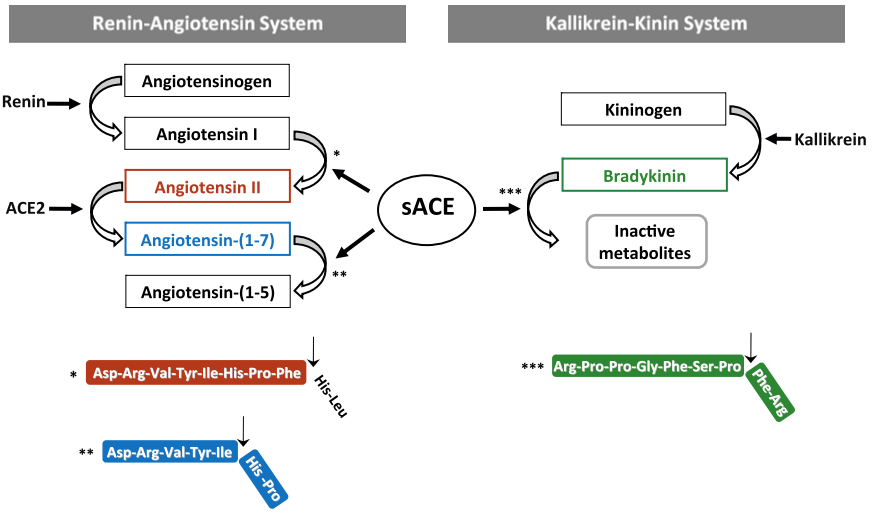

Fig. 1. Role of sACE in the renin-angiotensin and kallikrein-kinin systems.

Haralambos Gavras who set out to study the effects of Ang II on systemic blood pressure and coronary and renal circulation. Together with Brunner et al., he found that by administering parenterally *Bj*-BPP-9a (teprotide, <Glu–Trp–Pro–Arg–Pro–Gln–Ile–Pro–Pro) to hypertensive patients, the arterial blood pressure dropped significantly (Gavras et al., 1974, 1978). In spite of being essential to demonstrate that sACE inhibitors could represent a new class of antihypertensive drug, the snake venom peptide was not developed as a drug, mainly because it was not effective by oral route.

The design of a new class of sACE inhibitors, captopril, not presenting the drawbacks of the venom peptides, was based on a hypothetical working model which had sACE as a zinc metallopeptidase with the catalytic center of carboxypeptidase A. In this model, the interaction of the peptide-substrate/inhibitors with sACE corresponded to the terminal amino acid sequence Phe-Ala-Pro (analog of *Bj*-BPP-5a). This work led to the proposal that the venom peptides were substrate analogs that bound competitively to the substrate-binding sites (Cushman and Ondetti, 1999). Another important insight for the design of captopril derived from the work of Byers and Wolfenden (1973) who described a new concept for inhibitors of carboxypeptidase A based on benzylsuccinic acid. Cushman and Ondetti recognized that part of the binding affinity of benzylsuccinic acid derived from the coordination of the zinc ion at the active site with the carboxyl of the benzylsuccinic group. Extensive structure–activity studies showed that the simple structure of the Ala–Pro analog (D-2-methylsuccinyl-L-proline) was optimal for binding to the zinc-binding carboxyl group of sACE. Replacement of the carboxyl by a sulfhydryl group (coincidentally suggested by Ferreira and Rocha e Silva, 1962, and Erdös and Wohler, 1963) enhanced the inhibitory activity of the molecule by 1000-fold, yielding captopril (Fig. 2). This compound proved to be one of the most potent competitive inhibitors of sACE, and, most importantly, the first truly useful antihypertensive drug designed to bind to the active sites of this enzyme.

The development of captopril, which occurred in the late 1970s and early 1980s became a paradigm for "rational drug design", a concept that is much heralded today as something made possible by computer imaging and

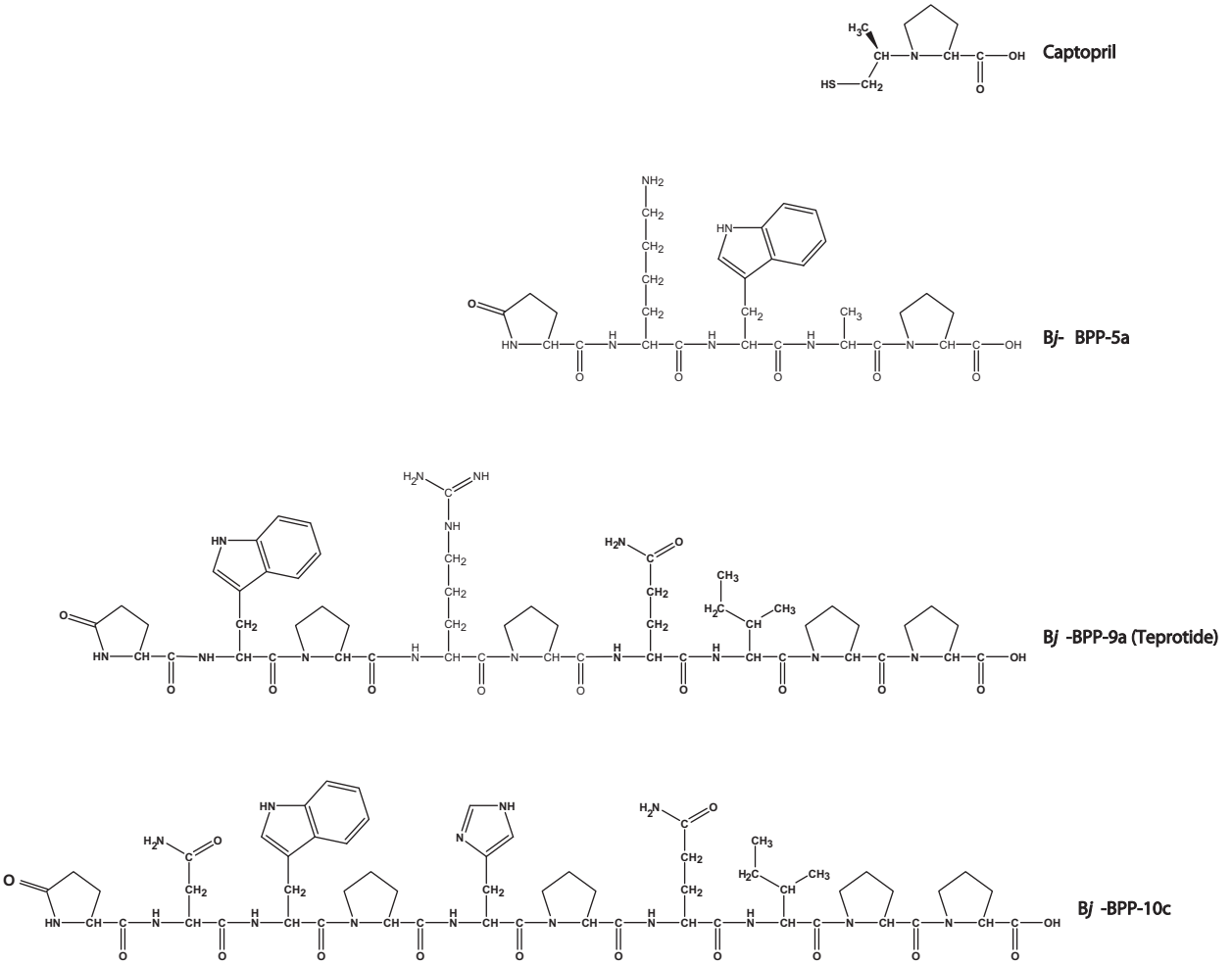

Fig. 2. Structures of captopril and *Bj*-BPPs.

genome science (van Dongen et al., 2002). The conception of captopril structure was certainly based on a series of brilliant insights that included the use of carboxypeptidase A as an enzymatic model, the determination of structural features of BPPs and the influence of thiol groups for sACE inhibition. Captopril was a blockbuster drug and inspired the creation of several generations of similar antihypertensive compounds.

Millions of hypertensive patients worldwide, who are treated with sACE inhibitors, benefit from the widely recognized pioneer studies of Rocha e Silva and Ferreira et al. who, ultimately, revealed the pivotal role played by Ang II, Bk and sACE in the regulation of the vascular tonus (Acharya et al., 2003). The success of captopril design and its clinical application led Cushman and Ondetti to share the 1999 Albert Lasker Award in Clinical Medical Research.

3. Targeting the C-domain active site of sACE

It is now known that sACE is unrelated to the carboxypeptidase A class of enzymes, as had been suggested (Cushman et al., 1977), but instead, it falls into the group of the metallopeptidases, characterized by two tandem active sites, containing the sequence HEXXH, a zinc-binding motif (Vallee and Auld, 1990). It is a transmembrane ectoprotein whose homologous active sites are located at the N- and C-domains of the protein. Some differences in catalytic properties have been observed for these two sites. For instance, the site at the N-domain (N-site) is notably more active toward the hemoregulatory peptide *N*-acetyl-Ser–Asp–Lys–Pro (AcSDKP) (Rousseau et al., 1995) and the hormone LHRH (Ehlers and Riordan, 1991), besides being the most sensitive for inhibition by captopril (Wei et al., 1992). Crystallographic data indicate that the active sites of tACE (testicular ACE) are located in a deep cavity. The amino-terminal helices form a lid-like extension that partially covers the active site channel limiting the access to substrates and inhibitors, thus requiring a special structure of substrate/inhibitor to enter the channel (Natesh et al., 2003). This is the most peculiar enzymatic feature of the oligopeptidases (Camargo et al., 1979, 1997; Fülöp et al., 1988). Therefore, in order to affect a specific site of sACE, the structural requirements for a substrate/inhibitor ought to be remarkable. In fact, these structural requirements were found among the *Bj*-BPPs (Cotton et al., 2002), as shown for the decapeptide *Bj*-BPP-10c (<Glu–Asn–Trp–Pro–His–Pro–Gln–Ile–Pro–Pro), which is approximately 400-fold more selective for the active site at the C-domain ($K_i = 0.5$ nM), than for the N-domain ($K_i = 200$ nM). An opposite result was observed for the *Bj*-BPP-12b (<Glu–Trp–Gly–Arg–Pro–Pro–Gly–Pro–Pro–Ile–Pro–Pro), which displays a K_i value of 5 nM for the ACE N-domain and is 30-fold less potent at the C-domain (Hayashi et al., 2003). Of particular clinical interest is the fact that, *in vivo*, Ang I is predominantly hydrolyzed by the C-site, whereas Bk is hydrolyzed by both active sites (Junot et al., 2001). Hence, a purely C-site selective inhibitor would be beneficial in two aspects: i) it would reduce the vasopressor effect of Ang II by inhibiting its degradation by the C-site; and ii) the preserved activity of the N-site would reduce the Bk accumulation that is likely responsible for side effects of the sACE inhibitors such as, bradykinin-mediated angioedema (Messerli and Nussberger, 2000). Thus, the extraordinary selectivity of *Bj*-BPP-10c for the C-site of sACE could be particularly valuable for the structure-guided drug design aiming at the development of selective inhibitors for each domain of sACE, which could be therapeutically desirable (Acharya et al., 2003).

4. The BPPs as putative endogenous bioactive peptides

The molecular evolution of each venom toxin seems to have been necessary for the toxicological performance of the venom, which relies on in the synergistic effect of all toxins resulting in the symphony of envenoming characterized by the rupture of the systemic homeostasis of the prey. On the other hand, it has been shown that the structural design of several known toxins evolved from endogenous molecules of the venomous animal (Fry et al., 2006) derived from clearly distinct architectural motifs, in order to adapt to different targets such as ion channels, enzymes and receptors, thus defining a special strategy of envenoming (Lewis and Garcia, 2003). The biodiversity of animal toxins, particularly concerning venom peptides, make them a unique source of leads and structural templates, from which new therapeutic agents could be developed. A classical example is captopril.

As described above, the *Bj*-venom targets the cardiovascular system of the prey not only by the action of kallikrein-like serine proteases but also by the effect of *Bj*-BPPs. Specifically, *Bj*-venom presents several BPPs (Ianzer et al., 2004; Zelanis et al., 2010), however, it is not fully known whether the cardiovascular effects of different proline-rich peptides are exclusively due to the inhibition of sACE. These considerations provided the focus for the molecular biology studies of the snake proline-rich oligopeptides, aiming to relate the structure of its precursor with the known proteins/peptides participants of the cardio circulatory physiology.

Interestingly, the genetic origin of these peptides revealed the sequences of seven proline-rich oligopeptides expressed in tandem within the N-terminal portion of the C-type natriuretic peptide-precursor protein in the *Bj*-venom gland (Murayama et al., 1997) and the *Bj*-CNS (Hayashi et al., 2003) (Fig. 3). The BPPs contained in the *Bj*-brain precursor are strong *in vitro* inhibitors of sACE, showing K_i values in the nanomolar range, and also potentiate the Bk effects as observed in smooth-muscle bioassays, and *in vivo* experiments (Hayashi et al., 2003). *In situ* hybridization studies revealed the presence of *Bj*-BPP precursor mRNAs in distinct regions of the *Bj*-brain. The biochemical and the cardiovascular activities of the brain *Bj*-BPPs (Hayashi and Camargo, 2005) and C-type natriuretic peptide (Rubattu et al., 2008), their presence within the same precursor of the neuroendocrine regulator C-type natriuretic peptide, and their expression in regions of the snake brain, correlated to neuroendocrine functions, strongly suggest that these peptides belong to a novel class of endogenous bioactive peptides (Hayashi and Camargo, 2005).

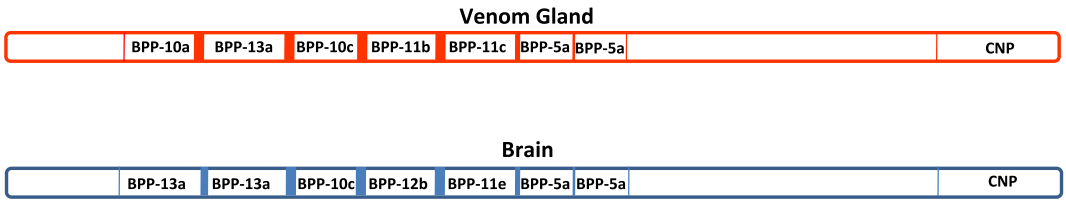

Fig. 3. Schematic of cDNAs encoding the CNP-precursor proteins containing seven *Bj*-BPPs.

5. Argininosuccinate synthetase (AsS), an unexpected target for the antihypertensive action of *Bj*-BPP-10c

The understanding of the entire therapeutic potential of snake venom sACE inhibitors has been hindered by the assumption that their biological activity is fully embodied by captopril, the prototype sACE active-site directed inhibitor. As we will describe below, this assumption is far from the real biological activities displayed by these peptides. In fact, the dissociation between the biological effect of the BPPs and inhibition of sACE had long been hypothesized (Camargo and Ferreira, 1970; Greene et al., 1972). The efforts to understand the antihypertensive effect of *Bj*-BPP-10c in spontaneously hypertensive rats (SHR) corroborated our previous hypothesis. Indeed, studies on the effects of *Bj*-BPP-10c at low doses ranging from 0.47 to 71 nmol/kg showed that the peptide is able to produce a potent and long-lasting reduction of mean arterial pressure in SHR, without affecting sACE activity (Ianzer et al., 2007). However, these studies also revealed an intriguing result: the peptide had no hypotensive effect by itself in normotensive rats (Ianzer et al., 2007). These results prompted us to investigate whether *Bj*-BPP-10c had another target besides sACE. Two reasons led us to search the kidney for the other putative target: i) the crucial role played by the kidney in the control of arterial blood pressure (Skeggs et al., 1981); ii) the selective concentration and long-lasting permanence of ^{125}I *Bj*-BPP-10c in the mouse kidney, even when a saturating concentration of captopril was administered with the peptide (Silva et al., 2008).

Affinity chromatography, using immobilized *Bj*-BPP-10c, associated with mass spectrometric and immunoblot analyses, allowed the identification of AsS as the main ligand for the peptide in the kidney cytosol. More importantly, *in vitro*, *Bj*-BPP-10c enhanced the affinity of the enzyme for two of its three substrates: the *Km* value for ATP and citrulline decreased 4.4- and 2.0-fold, respectively in the presence of 0.5 μM *Bj*-BPP-10c, thus promoting AsS activation (Guerreiro et al., 2009).

In mammals, AsS, together with argininosuccinate lyase (AsL), is part of the urea cycle in the liver and of the arginine–citrulline cycle, the major source of arginine and nitric oxide in renal and endothelial cells, respectively (Husson et al., 2003; Ye et al., 2007) (Fig. 4). These enzymes are rate-limiting components in both the urea- and the arginine–citrulline cycles. It is recognized that the impaired biosynthesis of arginine affects a number of metabolic and signaling pathways, such as the generation of a wide range of biologically active intermediates, e.g. nitric oxide, polyamines, creatinine and L-amino acids (Wu and Morris, 1998). In endothelial cells, the arginine–citrulline cycle, together with nitric oxide synthetase (NOS), ensures the supply of NO, which plays many roles as a signaling molecule in mammalian physiology, including the regulation of the vascular tonus. Since the peptide-induced NO metabolites production was impaired by both MDLA (α-methyl-DL-aspartic acid) and L-Name (N^G-nitro-L-arginine methyl ester), specific inhibitors of AsS and NOS, respectively, we were able to confirm that *Bj*-BPP-10c activated AsS, and that NOS was involved in the increase of NO metabolite production by HUVECs (Guerreiro et al., 2009) (Fig. 4).

An additional property of *Bj*-BPP-10c turns this peptide into an attractive potential lead molecule for drug development: it is able to penetrate cells, where it remains as an intact molecule at least for two hours (Guerreiro et al., 2009), which likely explains the long-lasting effect of this peptide in SHR (Ianzer et al., 2007).

One of the most intriguing questions concerning the antihypertensive effect of *Bj*-BPP-10c is the fact that it only causes sustained reduction of mean arterial pressure in SHR, but not in normotensive rats. The increased output of NO by *Bj*-BPP-10c through the arginine-dependent NO producing cells of SHR appears to be part of the explanation, and accordingly, the antihypertensive effect of *Bj*-BPP-10c was impaired by MDLA (Guerreiro et al., 2009). The importance of the arginine–citrulline cycle for endothelial NO production was supported by a report of two infants with a deficiency of AsL, who were shown to be hypertensive (Fakler et al., 1995).

According to our study, *Bj*-BPP-10c not only increases the plasma level of arginine in SHR, but also in normotensive rats (Guerreiro et al., 2009). Why then, did it not cause a reduction of the blood pressure in normotensive animals? The answer might be related to the regulation of the citrulline/arginine recycling activity for NO production in endothelial cells. It is known that the level of this activity, which occurs in the caveolae of endothelial cells (Solomonson et al., 2003), is lower in SHR as compared to normotensive rats. The increase of the caveolar AsS activity might, consequently, be translated into enhancement of the low production of NO by the endothelial cells of SHR (Hasegawa et al., 1992). This hypothesis seems plausible since the recycling enzymes, AsS and AsL, co-localize with NOS in the caveolae (Solomonson et al., 2003). Moreover, it has been reported that the expression of the AsS gene is decreased at the onset of hypertension in SHR (Koeners et al., 2007). The putative dysfunction of AsS activity in SHR could be specifically corrected by AsS activation, thus providing more arginine

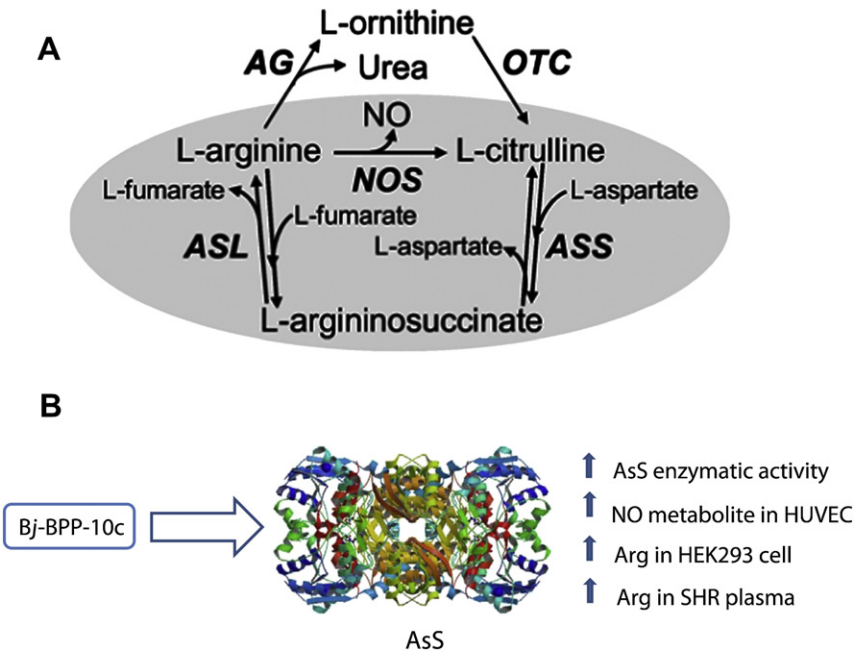

Fig. 4. (A) A simplified scheme of the urea and arginine–citrulline cycles (shadowed). AG: arginase; OTC: ornithine transcarbamylase; NOS: nitric oxide synthase; AsS: argininosuccinate synthetase; AsL: argininosuccinate lyase. Reprinted from Ye et al. (2007) with permission from Wiley–Blackwell. (B) Effects of *Bj*-BPP-10c upon AsS activity. AsS structure is PDB ID: 2nz2 (Karlberg et al., 2008).

to the NOS, and consequently reducing the arterial blood pressure of these animals.

Another possible target for the *Bj*-BPP-10c would be the central nervous system (CNS), since the labeled decapeptide aforementioned was also found in the mouse brain following systemic administration (Silva et al., 2008). It suggests that *Bj*-BPP-10c might across blood brain barrier thus provoking centrally mediated effects. NO has been attributed to reductions in arterial pressure and signal transmission in the CNS (Garthwaite and Boultn, 1995). NO-mediated actions in the CNS include the control of the vasomotion (Patel et al., 2001) and the baroreflex control of heart rate (Stauss and Persson, 2000). Recently, improvements in the sensitivity of the baroreflex in SHR through a central NO-dependent mechanism were reported after *Bj*-BPP-10c injection (Lameu et al., 2010a,b). Indeed, *in vitro*, *Bj*-BPP-10c changes the release of the neurotransmitters GABA and glutamate (Lameu et al., 2010a,b), besides enhancing citrulline-NO cycle in astroglioma cells (Oliveira et al., 2010). Altogether, these neuronal effects described for the *Bj*-BPP-10c may accomplish modulation of several functions as autonomic, behavioral and others.

Bj-BPP-10c enhances arginine metabolism, a natural precursor molecule for NO synthesis, which has its own levels subjected to very precise mechanisms of physiological control. For this reason we believe that *Bj*-BPP-10c is a more suitable molecule to regulate the increase of NO production in NO deficiency pathologies as compared to NO donors (Worcel, 2005) mainly because the effect of the latter compounds is not subjected to physiological control, thus being more susceptible of generating undesired reactive by-products (Ridnour et al., 2004). In line with the ongoing trend of toxin-inspired drug development (Fox and Serrano, 2007), we believe that *Bj*-BPP-10c could be considered a lead molecule for the development of therapeutic agents for the treatment of various diseases related to NO deficiency, as cause or effect. Therefore, we propose AsS as a novel target for the controlled modulation of blood pressure.

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Conflict of interest

The authors declare no conflict of interest regarding the content of this manuscript.

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