Bothrojaracin, a *Bothrops jararaca* Snake Venom-Derived (Pro)Thrombin Inhibitor, as an Anti-Thrombotic Molecule

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Key Words

Bothrojaracin · Snake venom · Anti-thrombotic effects · Thrombin · Prothrombin

Abstract

Bothrojaracin (BJC) is a selective and potent thrombin inhibitor ($K_D = 0.6$ nM) which also binds to prothrombin on proexosite I ($K_D = 175$ nM). Incubation of BJC with human or rat plasma produced a band that co-migrates with purified prothrombin-BJC complex. We further analyzed the in vivo anti-thrombotic effect of BJC on a venous thrombosis model in rats that combines stasis and hypercoagulability. The administration of 1 mg/kg (i.v.) doses of BJC decreased thrombus weight by ~95%. Evaluation of the in vivo effect of BJC in mice using a pulmonary thromboembolism model induced by thrombin showed that BJC protects 100% of mice from death. Altogether, our data show that BJC is a potent anti-thrombotic agent that could further help the development of new prothrombin-directed drugs.

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Introduction

Venoms from different species of snakes contain components, mainly proteins and peptides, which interfere in various physiopathological processes, including neurotransmission, growth and metastasis of tumoural cells, inflammation, immune responses, cell growth, apoptosis

and haemostasis [1]. A large variety of active principles that interfere in one or more physiological process have been isolated and their mechanism of action characterized [2, 3].

Bothrojaracin (BJC) is one of those proteins and it was described in 1993 as the first specific thrombin inhibitor isolated from the *Bothrops jararaca* snake venom [4]. Since then its structure and mechanism of action has been studied and characterized by our group and that of Dr. C. Bon (Pasteur Institute, France) [5].

Structural Features

BJC is a 27-kDa protein with an acidic pH of 4.2, formed by two distinct chains linked together by a single disulfide bridge [4]. Determination of its full amino-acid sequence (deduced from cDNA) showed that BJC is a member of the C-type lectin-like family [5]. BJC chains (A and B) are encoded by two distinct mRNAs showing high degree of homology between each other and to a large number of molecules isolated from snake venoms: botrocetin (80 and 60% homology to chains A and B, respectively); factors IX/X-binding protein (57 and 54%); GP Ib-binding protein (65 and 62%); and salmorin (56 and 52%), among others [6–9].

As seen for other C-type lectin-like proteins, BJC displays only 11 out of the 13 amino-acid residues that constitute the Carbohydrate Recognition Domain (CRD), being unable to bind to carbohydrates [5]. In addition,

analysis of Ca^{2+} -binding sites found in other lectin-like proteins [10] strongly suggests that none of BJC subunits recognize this ion. In fact, Ca^{2+} ions are not required for BJC activity (unpublished data).

Bothrops jararaca as well as other *Bothrops* sp. produce a large number of BJC isoforms that differ in their primary sequence [11–14]. These data reveal that there is a family of BJC-like molecules, isoforms that are present in the venom of all *Bothrops* sp. studied so far. Interestingly, common epitopes are also shared with high-molecular-weight lectin-like molecules such as convulxin [15].

Mechanism of Action

BJC interacts with thrombin, forming a stable 1:1 complex, with an estimated K_D for immobilized BJC of 0.6 nM [4, 16]. Competition assays showed that BJC displaces thrombin ligands such as fibrin, hirudin and thrombomodulin, indicating that it interacts with thrombin through the so-called thrombin binding exosite I. In addition, BJC inhibits the activation of factor V by thrombin, and is therefore a potent inhibitor of the feedback activation of the clotting cascade [17]. BJC may also bind to γ -thrombin, a proteolytic derivative of α -thrombin that lacks exosite I. This interaction, that shows a 10-fold lower affinity, is disrupted by heparin indicating that it is mediated by thrombin exosite II [16].

Remarkably, the catalytic activity of thrombin towards chromogenic substrates is not inhibited by BJC [4]. In fact, fluorescence assays showed that this protein produces distinct structural changes in the thrombin catalytic site, depending on whether it interacts with exosite I or exosite II [18]. Thus, BJC enhances α -thrombin activity towards some chromogenic substrates, while it decreases the hydrolysis of the same substrates by γ -thrombin.

Moreover, BJC can also displace the thrombin that is bound to the clot and also can inhibit its clotting activity, suggesting that it may be an important tool to impair growth of thrombi [16].

BJC can also bind with high specificity to the thrombin zymogen prothrombin [16, 19, 20]. It binds to prothrombin with a K_D of 175 nM, indicating a ~ 100 -fold lower binding affinity than that found for thrombin. Peptides based on the C-terminal of hirudin have been also shown to bind prothrombin with ~ 100 -fold lower affinities when compared to thrombin [21]. Actually, BJC competes with Hirudin^{54–65} for binding to prothrombin [22], demonstrating that both molecules bind to the so-called proexosite I of prothrombin [21].

Fig. 1. Anti-coagulant mechanism of BJC. **A** Formation of regular prothrombinase complex. **B** Mechanism 1 of BJC inhibition is based on its binding to thrombin exosite-I and consequent inhibition of its activities. **C** Mechanism 2 is based on the inhibition of prothrombin activation through interaction with proexosite I. BJC = Bothrojaracin; PT = prothrombin; Va = activated factor V; Xa = activated factor X; T = thrombin.

Table 1. Effects of bothrojaracin in vivo

Experiments, doses 1 mg/kg	Animals	Effects	References
Deep venous thrombosis, in vivo ^a	Wistar rats weighing ~160 g (n = 3)	95% inhibition	26
Clotting effect – PT, ex vivo ^b	Wistar rats weighing ~160 g (n = 5)	1.7-fold over control	26
Clotting effect – aPTT, ex vivo ^b	Wistar rats weighing ~160 g (n = 5)	1.5-fold over control	26
Hemorrhagic effect, in vivo ^c	Wistar rats weighing ~160 g (n = 5)	6.0-fold over control	27
Thromboembolism, in vivo ^d	Balb-C mice weighing 20 g (n = 15)	100% protection	28

^a Deep vein thrombosis model: Thrombus formation was induced by a combination of stasis and hypercoagulability at vena cava produced by intravenous administration of thromboplastin. After 20 min the formed thrombus was detached from the segment, dried for 1 h at 60°C and weighed. Bothrojaracin was administered 5 min before thrombogenic stimulus.

^b Ex vivo determination of APTT and PT: For APTT tests, cephalin plus kaolin (APTT reagent) were incubated for 1 min with 50 µl of plasma (37°C). The reaction was started by addition of 100 µl of CaCl₂ (25 mM). For PT tests, 50 µl of plasma were incubated for 2 min (37°C). The reaction was started by addition of 100 µl of thromboplastin with calcium (PT reagent).

^c Rat tail-transection model: For evaluation of the bleeding effect, the carotid artery was exposed and dissected. Bothrojaracin was administered through silicone tubing inserted into the carotid artery. Immediately 5 min after bothrojaracin administration the rat's tail was cut 3 mm from the tip. Blood loss was evaluated 120 min later as a function of absorbance at 540 nm due to the hemoglobin content in water solution.

^d Thromboembolism model: Thrombotic event was induced by human thrombin (2,000 IU/kg animal), and the survival rate was evaluated 15 min after thrombotic event. Bothrojaracin was injected i.v. at retro-orbital venous plexus 60 min before the thrombogenic stimulus. Results shown represent 3 groups of 5 animals each.

Formation of the BJC-prothrombin complex is particularly effective in preventing thrombin formation catalyzed by *Oxyuranus scutellatus* venom [20], which contains a highly active prothrombin activator structurally related to FXa/FVa [23]. We have also demonstrated that prothrombin activation by the prothrombinase complex is decreased in the presence of either BJC or Hirudin^{54–65}. This effect was much more evident in the presence of factor Va and activated platelets, suggestive of a physiological role for proexosite I [24].

Altogether, we have concluded that BJC exerts its anticoagulant effect by two distinct mechanisms (fig. 1): it binds to activated thrombin through exosite 1, blocking fibrinogen clotting, platelet activation, factor V activation and other effects, and it interacts with prothrombin, decreasing its proteolytic activation – especially in the presence of factor Va [19, 20, 24]. These characteristics make BJC a promising tool for the investigation of anti-thrombotic activities in vivo.

In vivo Effects of Bothrojaracin

We have analyzed the in vivo anti-thrombotic effects of BJC in rats by using a venous thrombosis model that combines stasis and hypercoagulability. Thromboplastin

(3 mg/kg) was injected into the vena cava (below the distal loose suture) and stasis was immediately established, after 20 min the thrombus was removed. The formed detached thrombus was blotted on filter paper, dried, and weighed (8.1 ± 0.8 mg). Intravenous administration of BJC (1 mg/kg), 5 min before thrombosis induction, caused a significant decrease of ~95% in thrombus weight. The same dose of BJC conferred 100% protection against thrombin-induced mortality in mice, on a pulmonary thromboembolism model (see table 1 for references). On the other hand, BJC produced an important increase in the bleeding effect in rats (tail-transection model), although with very little changes on ex vivo clotting times (aPTT and PT; table 1).

Compared to other anti-thrombotic drugs such as heparin [25] or glycyrrhizin [26], BJC shows a prolonged effect. In fact we have evidence that BJC remains in the plasma bound to prothrombin for at least for 12 h in vivo (unpublished results). Altogether, our data show that BJC is a potent anti-thrombotic agent and may be useful for the development of new drugs directed to both prothrombin and thrombin. On the other hand, prothrombin concentration in plasma (100–150 mg/ml) is very high when compared to other clotting factors. This is a possible limiting issue for the use of prothrombin inhibitors. Even though, efficacy and pharmacokinetics studies will be im-

portant in order to determine which pathological state might be treated and/or prevented with anti-thrombotic drugs displaying this particular mechanism of action.

Conclusion

Although the mechanism of action of BJC is known in considerable detail, the structure-function relationship is yet to be understood. The structure of BJC complexed with prothrombin and thrombin will certainly furnish the necessary information that is missing today and which is

necessary to understand why and how BJC binds to thrombin and prothrombin. This is a crucial issue to understand prior to use of this molecule as a prototype for the development of new drugs that can bind to zymogens such as prothrombin.

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