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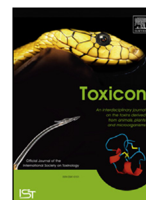

# Interaction of *Bothrops jararaca* venom metalloproteinases with protein inhibitors

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ABSTRACT

Snake venom metalloproteinases (SVMPs) play important roles in the local and systemic hemorrhage observed upon envenomation. In a previous study on the structural elements important for the activities of HF3 (highly hemorrhagic, P-III-SVMP), bothropasin (hemorrhagic, P-III-SVMP) and BJ-PI (non-hemorrhagic, P-I-SVMP), from *Bothrops jararaca*, it was demonstrated that they differ in their proteolysis profile of plasma and extracellular matrix proteins. In this study, we evaluated the ability of proteins DM43 and α2-macroglobulin to interfere with the proteolytic activity of these SVMPs on fibrinogen and collagen VI and with their ability to induce hemorrhage. DM43 inhibited the proteolytic activity of bothropasin and BJ-PI but not that of HF3, and was not cleaved the three proteinases. On the other hand, α2-macroglobulin did not inhibit any of the proteinases and was rather cleaved by them. In agreement with these findings, binding analysis showed interaction of bothropasin and BJ-PI but not HF3 to DM43 while none of the proteinases bound to α2-macroglobulin. Moreover, DM43 promoted partial inhibition of the hemorrhagic activity of bothropasin but not that of HF3. Our results demonstrate that metalloproteinases of *B. jararaca* venom showing different domain composition, glycosylation level and hemorrhagic potency show variable susceptibilities to protein inhibitors.
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## 1. Introduction

Manifestations of local and systemic damage, such as hemorrhage and coagulopathy, are among the most dramatic effects of envenomation by viperid snakes. In cases of less severe envenomation, the hemorrhagic effect is usually localized at the site of the bite. However, hemorrhage can be also found spread widely through a substantial area of the involved extremity and, in cases of severe envenomation, bleeding in organs distant from the site of bite, such as heart, lungs, kidneys and brain, may also occur (Gutierrez et al., 2005). Snake venom metalloproteinases (SVMPs) are abundant toxins in viperid venoms and play important roles in the severe local tissue damage, hemorrhage and profound consumption coagulopathy observed upon envenomation (Gutierrez et al., 2005; Fox and Serrano, 2008). They are classified in three classes according to their domain organization and the proteinase domain of hemorrhagic toxins is believed to function to degrade capillary basement membranes, endothelial cell surface proteins, and stromal matrix ultimately causing extravasation of capillary contents into the surround stroma (Baramova et al., 1989; Escalante et al., 2009; Paes Leme et al., 2012). Interestingly, the P-III class of SVMPs is typically much more potent in causing hemorrhage compared with the P-I and P-II classes that lack the

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disintegrin-like/cysteine-rich domains. Proteolytic degradation of capillary structures allied to effects on plasma proteins and platelet aggregation have been considered to be the key features underlying the hemorrhagic potency of P-III SVMP hemorrhagic toxins (Baldo et al., 2010; Serrano et al., 2007; Rucavado et al., 2005; Santoro and Sano-Martins, 2004; Wijeyewickrema et al., 2007).

Three SVMPs of different domain organization and glycosylation levels, isolated from *Bothrops jararaca* venom, were shown to display different proteolytic and hemorrhagic activities: HF3 (P-IIIa class) is highly glycosylated showing a 70 kDa molecular mass (Silva et al., 2004) and is an extremely hemorrhagic toxin that shows a minimum hemorrhagic dose of 240 fmol on the rabbit skin (Assakura et al., 1986); bothropasin (P-IIIb class), which contains a minor carbohydrate moiety, is ~80 times less hemorrhagic than HF3 but is a highly proteolytic enzyme (Assakura et al., 2003), and BJ-PI (P-I class) is a potent fibrinogenolytic enzyme but is devoid of hemorrhagic activity (Oliveira et al., 2009). In a recent comparative study of the structural elements important for the activities of HF3, bothropasin and BJ-PI, it was demonstrated that SVMPs of different domain composition, glycosylation level and hemorrhagic potency differ in their interaction affinities and proteolytic specificity upon plasma and extracellular matrix proteins (Oliveira et al., 2010).

The inhibition of SVMP activity may be an important factor in the control of local and systemic effects of envenomation. Despite the general success of antivenom therapy, it is still important to find natural inhibitors of SVMPs and to design synthetic inhibitors to be used as therapeutic tools. The anti-hemorrhagic protein DM43, isolated from the serum of the opossum *Didelphis marsupialis*, was able to inhibit the hydrolysis of casein, fibronectin and fibrinogen by *B. jararaca* venom proteinases and the hemorrhagic effect of jararhagin (Neves-Ferreira et al., 2000). The mechanism of such inhibition involves the formation of soluble inactive complexes between the serum protein and the toxin (Neves-Ferreira et al., 2002). On the other hand, the partial or complete resistance of some SVMPs to the inhibitory action of plasma proteinase inhibitors, especially the macroglobulins, is believed to be an important factor contributing to the variable capacity of SVMPs to induce local and systemic hemorrhage (Baramova et al., 1990; Kamiguti et al., 1994). Although α2-macroglobulin is able to form covalent complexes with the proteinases, a number of P-III class SVMPs are not inhibited by this protein (Baramova et al., 1990; Escalante et al., 2003).

In order to further analyze the features of P-I and P-III SVMPs involved in their proteolytic and hemorrhagic activities, this study focused on the inhibition HF3, bothropasin and BJ-PI by proteins DM43 and α2-macroglobulin. Our results demonstrate that HF3, bothropasin and BJ-PI have different susceptibilities to inhibition by proteins.

## 2. Materials and methods

### 2.1. SVMPs

HF3, bothropasin and BJ-PI were isolated from *B. jararaca* venom as described previously (Oliveira et al., 2009). Partially *N*-deglycosylated HF3 was prepared as described elsewhere (Oliveira et al., 2010).

### 2.2. Protein inhibitors

Protein DM43 was isolated according to Neves-Ferreira et al. (2000) and α2-macroglobulin was purchased from Sigma (St. Louis, MO).

### 2.3. Effect of DM43 and α2-macroglobulin on the fibrinogenolytic activity of HF3, bothropasin and BJ-PI

Native and partially *N*-deglycosylated HF3 (200 ng), or bothropasin (200 ng) were preincubated with protein DM43 (200 ng) and BJ-PI (200 ng) was preincubated with DM43 (400 ng) in 50 mM Tris–HCl, pH 8.0, containing 1 mM $CaCl_2$ for 40 min at room temperature and then incubated with human fibrinogen (10 μg) for 1 h at 37 °C.

Native and partially *N*-deglycosylated HF3 (200 ng), bothropasin (200 ng) and BJ-PI (200 ng) were preincubated with α2-macroglobulin (2 μg) in 50 mM Tris–HCl, pH 8.0, containing 1 mM $CaCl_2$ for 30 min at 37 °C and incubated with human fibrinogen (10 μg) for 1 h at 37 °C.

Human fibrinogen (Kabi Diagnostica, Sweden) was dissolved in 0.15 M NaCl at 10 mg/ml and incubated with and without enzyme under identical conditions as a control. Reactions were stopped by adding four fold concentrated Laemmli sample buffer and samples were submitted to SDS-PAGE using 9% or 12% polyacrylamide gels (Laemmli, 1970). Gels were silver stained.

### 2.4. Effect of DM43 and α2-macroglobulin on the proteolytic activity of HF3, bothropasin and BJ-PI on collagen VI

Native HF3 or bothropasin (100 ng) were preincubated with protein DM43 (100 ng) and BJ-PI (100 ng) was preincubated with DM43 (200 ng) in 50 mM Tris–HCl, pH 8.0, containing 1 mM $CaCl_2$ for 40 min at room temperature and then incubated with collagen VI (5 μg) for 3 h at 37 °C.

Native and partially *N*-deglycosylated HF3 (200 ng), bothropasin (200 ng) and BJ-PI (200 ng) were preincubated with α2-macroglobulin (2 μg) in 50 mM Tris–HCl, pH 8.0, containing 1 mM $CaCl_2$ for 30 min at 37 °C and then incubated with collagen VI (5 μg) for 3 h at 37 °C.

Collagen VI (Sigma, Saint Louis, MO) was dissolved in 0.25% acetic acid at 2 mg/ml and the acidic pH was neutralized with 1.5 M Tris–HCl, pH 8.8, prior to the incubation with and without enzyme under identical conditions as a control. Reactions were stopped by adding four fold concentrated Laemmli sample buffer and then submitted to SDS-PAGE using 8% polyacrylamide gels (Laemmli, 1970). Gels were silver stained.

### 2.5. Proteolytic activity of HF3, bothropasin and BJ-PI on DM43 and α2-macroglobulin

DM43 (500 ng) was incubated with HF3, bothropasin or BJ-PI (500 ng) in 50 mM Tris–HCl, pH 8.0 containing 1 mM $CaCl_2$ for 1 h at 37 °C and the reactions were stopped by adding four fold concentrated Laemmli sample buffer and then submitted to SDS-PAGE under non-reducing

conditions using a 12% polyacrylamide gel (Laemmli, 1970). Gels were silver stained.

α2-macroglobulin (2 μg) was incubated with HF3, bothropasin or BJ-PI (200 ng) in 50 mM Tris–HCl, pH 8.0 containing 1 mM $CaCl_2$ for 30 min at 37 °C and the reactions were stopped by adding four fold concentrated Laemmli sample buffer and then submitted to SDS-PAGE under reducing conditions using a 9% polyacrylamide gel (Laemmli, 1970). Gels were silver stained.

### 2.6. Surface plasmon resonance

Protein–protein interactions were assessed by surface plasmon resonance (SPR) with a BIAcoreT100 system. Immediately after sensorchip docking, the instrument was primed three times with HBS as running buffer (10 mM HEPES, 150 mM NaCl, pH 7.4) and the biosensor detector response was normalized (automated procedure). DM43 and α2-macroglobulin were covalently immobilized on the BIAcore CM-5 sensorchip (carboxymethylated dextran matrix) according to the manufacturer's instructions. Briefly, the CM-5 chip was activated with a 1:1 mixture of 0.4 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and 0.1 M N-hydroxysuccinimide for 7 min. DM43 in 10 mM sodium citrate, pH 4.0 was injected over the activated CM-5 chip at 25 °C. Remaining active groups on the matrix were blocked with 1 M ethanolamine/HCl, pH 8.5. Immobilization on CM-5 sensorchip resulted in an average surface concentration of 0.9 ng/mm2 for protein DM43 and 3.0 ng/mm2 for α2-macroglobulin. A flow chamber subjected to the immobilization protocol but without any addition of protein was used as a reference surface (blank) for each experiment. Samples of native and partially N-deglycosylated HF3, native bothropasin and native BJ-PI (0.3 μM and 1.0 μM) in 10 mM HEPES, 150 mM NaCl, pH 7.4 were injected over the immobilized surfaces and control surface at a flow rate of 30 μl/min for 1 min at 25 °C. Between experiments, the surfaces were strictly regenerated with a single pulse of 10 mM glycine-HCl, pH 2.5 for 1 min, followed by an extensive wash procedure using running buffer. All results were analyzed using Biaevaluation software (version 1.1.1) and sensorgrams were processed by subtracting data from the reference surface and adjusting the response on the *y*-axis (baseline). Individual experiments were performed three times.

### 2.7. Effect of DM43 on the hemorrhage induced by HF3 and bothropasin

Male Swiss mice (18–20 g) were obtained from Instituto Butantan and received water and food *ad libitum*. All the procedures involving mice were in accordance with the ethical principles in animal research adopted by the Brazilian Society of Animal Science and the National Brazilian Legislation no. 11.794/08. Protocols were approved by Institutional Animal Care and Use Committee (#688/09).

To evaluate the ability of protein DM43 to neutralize the hemorrhage induced by HF3 and bothropasin, groups of three mice were injected intradermally on the dorsal region with one of the following solutions: i) 100 μL of 0.15 M NaCl; ii) 100 μL of a solution containing 0.5 μg of HF3 in 0.15 M NaCl; iii) 100 μL of a solution containing 10 μg of bothropasin in 0.15 M NaCl; iv) 100 μL of a solution containing 20 μg of protein DM43; v) 100 μL of a solution containing 0.5 μg of HF3 preincubated with 0.7 μg of protein DM43; vi) 100 μL of a solution containing 10 μg of bothropasin preincubated with 20 μg of protein DM43. HF3 and bothropasin were preincubated with protein DM43 in 50 mM Tris–HCl, pH 8.0 containing 1 mM $CaCl_2$ for 30 min at 37 °C. After 2 h of injection the mouse dorsal skin was sectioned and the underside was photographed.

### 2.8. Homology modeling of HF3

With the purpose of investigating the spatial location of the putative N-glycosylation sites of HF3 (Genbank accession no. AF149788) in comparison with zinc metalloproteinase-disintegrin bothropasin (PDB ID: 3DSL), a tridimensional model of native HF3 was predicted using homology modeling using Modeller 9v2 program (Sali and Blundell, 1993) as described by Menezes et al. (2011). The modeled region included residues 193-603 according to the numbering of VAP1 structure (Takeda et al., 2006), which share 67% of sequence identity with bothropasin. The images were generated using PyMOL Molecular Graphics System, Version 1.2r3pre, Schrödinger, LLC (http://www.pymol.org).

## 3. Results and discussion

### 3.1. Effect of protein DM43 on the proteolytic activities of HF3, bothropasin and BJ-PI

DM43 is a 291 amino acid glycoprotein that possesses three Ig-like domains homologous to those found in the N-terminal region of $\alpha_1$B-glycoprotein (Neves-Ferreira et al., 2002). Here we tested the ability of DM43 to inhibit the cleavage of fibrinogen and collagen VI by HF3, bothropasin and BJ-PI (Fig. 1). The preincubation of the proteinases with DM43 resulted in the inhibition of the cleavage of fibrinogen by bothropasin and BJ-PI (Fig. 1A), which is in good agreement with a previous finding of the ability of DM43 to inhibit the fibrinogenolytic activity of jararhagin, a P-III class SVMP from *B. jararaca* venom, which shows high sequence similarity to bothropasin (Neves-Ferreira et al., 2000; Muniz et al., 2008), and botrolysin, a P-I class SVMP from *B. jararaca,* venom (Neves-Ferreira et al., 2002). However, the fibrinogenolytic activity of HF3 was not affected by DM43 (Fig. 1A). We then tested whether the large carbohydrate moiety of HF3 could prevent its interaction of DM43 since it contains five putative *N*-glycosylation sites (three are located at the proteinase domain and two at the cysteine-rich domain) (Silva et al., 2004). To evaluate the role of the carbohydrate moiety of HF3 in its ability to interact with DM43, we incubated it with *N*-glycosydase F under non-denaturing conditions. *N*-glycosydase F removes both complex and high mannose type *N*-linked oligosaccharides from proteins. In the case of HF3, when the incubation with this glycosidase is carried out under non-reducing conditions to avoid denaturation and the consequent loss of catalytic activity, it results in the partial deglycosylation of HF3, as illustrated by a shift of its

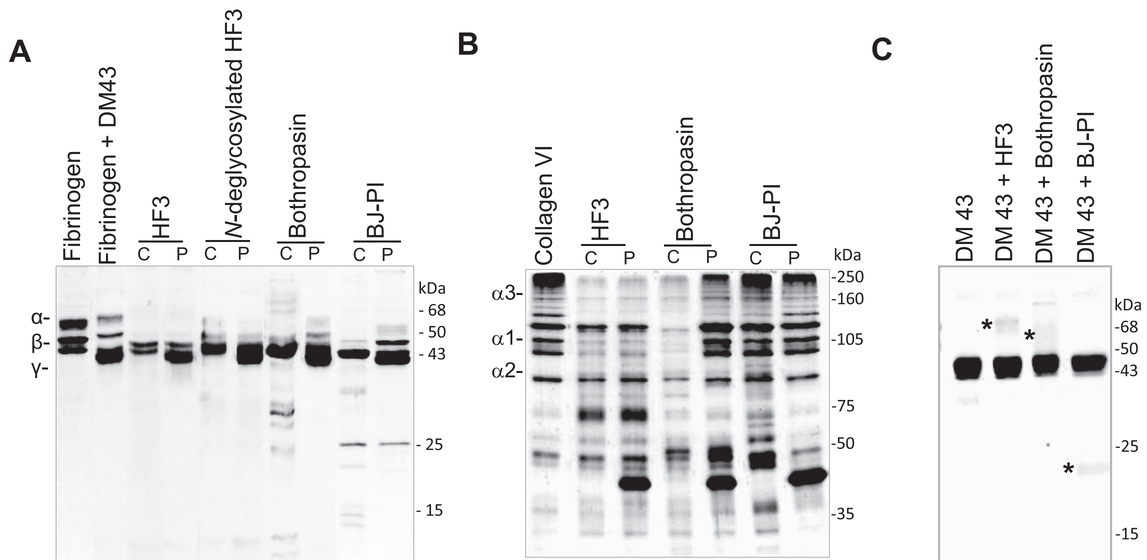


**Fig. 1.** Effect of the protein DM43 on the cleavage of fibrinogen and collagen VI by native and *N*-deglycosylated HF3, bothropasin and BJ-PI. The proteinases were preincubated with protein DM43 and then incubated with human fibrinogen (A) or with collagen VI (B); and protein DM43 was incubated with the proteinases (C), as described in the Materials and Methods section. Fibrinogen chains (α, β, and γ) and collagen VI chains (α1, α2 and α3) are indicated. * indicates the proteinase bands. C (control incubation). P (preincubation with DM43).

apparent molecular mass from 70 kDa to 60 kDa, and in the preservation of part of its fibrinogenolytic and hemorrhagic activities (Oliveira et al., 2010). However, the partial *N*-deglycosylation of HF3 has apparently not enabled the proteinase to interact with DM43 and hence the proteolysis of fibrinogen by *N*-deglycosylated HF3 was not inhibited by DM43 (Fig. 1A).

Furthermore, when we tested the ability of DM43 to inhibit the proteolytic activity of the metalloproteinases on collagen VI, bothropasin and BJ-PI interacted with DM43 as their activities were inhibited, while HF3 was not affected and clearly cleaved collagen VI in the presence of DM43 (Fig. 1B). We also submitted the protein DM43 to incubation with the three proteinases to check whether it would be degraded. Fig. 1C shows that DM43 remained intact after incubation with HF3, bothropasin and BJ-PI. Besides, under these conditions, no covalent interaction between the proteinases and DM43 was detected by SDS-PAGE under non-reducing conditions as the electrophoretic profile of DM43 did not change (Fig. 1C).

Considering the fact that the fibrinogenolytic and collagenolytic activities of bothropasin and BJ-PI were inhibited by DM43 while those of HF3 were not, we further explored the interaction of the three proteinases with DM43 by surface plasmon resonance (SPR) technology. Both bothropasin and BJ-PI (at 0.3 μM and 1.0 μM) bound in a concentration dependent fashion to a sensor chip on which DM43 had been immobilized (Supplemental Fig. 1). On the other hand, neither native HF3 nor partially *N*-deglycosylated HF3 showed any significant interaction with DM43. These results are consistent with the proteolytic assays and indicate that the inhibition of bothropasin and BJ-PI by DM43 is dependent of their physical interaction. The interaction of DM43 and jararhagin was recently shown to result in an equilibrium dissociation constant ($K_D$) of 0.33 nM (Brand et al., 2012). However, it has been previously shown that jararhagin C, a protein composed of the disintegrin-like and cysteine-rich domains of jararhagin did not form any complex with DM43, a fact that indicated the essential role of the metalloproteinase domain for the interaction of DM43 with SVMPs. Moreover, DM43 was not able to inhibit the fibrinogenolytic activity of atrolysin A, a highly glycosylated P-III class SVMP from *C. atrox* (Neves-Ferreira et al., 2002). Atrolysin A contains four putative glycosylation sites and shows a molecular mass of ~68 kDa by SDS-PAGE (Baramova et al., 1989), which is close to that of HF3. Considering these facts, a possible reason for the lack of inhibition of the fibrinogenolytic and collagenolytic activities of HF3 by DM43 would be the presence of the large carbohydrate moiety in this protein, which was only partially removed by deglycosylation under non-denaturing conditions.

Using the atomic coordinates for crystal structure of bothropasin (PDB ID: 3DSL) (Muniz et al., 2008), we modeled the structure of HF3 (Menezes et al., 2011) and analyzed site-directed mutations in its disintegrin-like and cysteine-rich domains. Here we used the modeled structure to analyze the positions of the five glycosylation sites in HF3 in comparison to the only one present in the structure of bothropasin (Asn373), which is located near the zinc binding sequence and is conserved in HF3 and bothropasin and in other SVMPs (Fox and Serrano, 2008) (Fig. 2). The interface between DM43 and jararhagin in their complex is unknown, however, stoichiometry results indicated that one molecule of jararhagin interacts with one monomer of DM43; moreover, DM43 behaves as a homodimer in solution and requires a molecular trigger (i.e. toxin binding) to dissociate (Brand et al., 2012). In the model of HF3 structure, four glycosylation sites are located in the metalloproteinase domain (Asn259 and Asn313) and

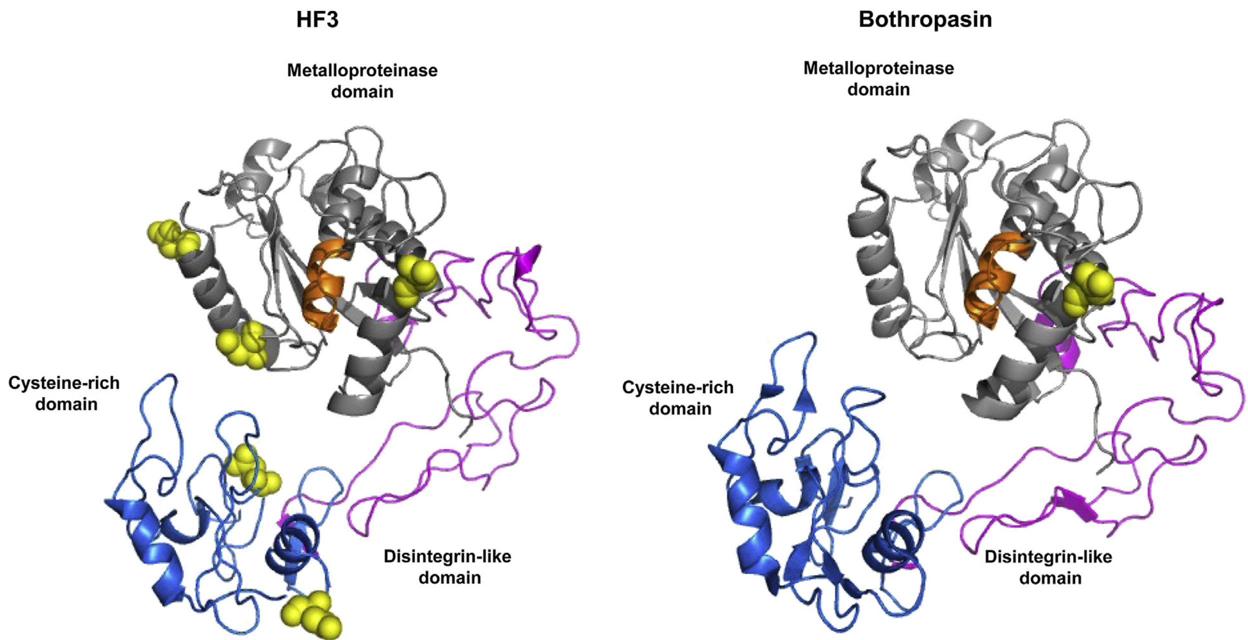


**Fig. 2.** Cartoon representations of the predicted structure of HF3 using molecular modeling and of the tridimensional structure of bothropasin (PDB code 3DSL). The overall structure shows the metalloproteinase (gray, with the zinc binding region in orange), disintegrin-like (magenta) and cysteine-rich (blue) domains. The putative N-glycosylation sites Asn259, Asn313 and Asn373, in the metaloproteinase domain, and Asn518 and Asn588 in the cysteine-rich domain, are shown as a spheres representation in yellow. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

in the cysteine-rich domain (Asn518 and Asn588) in flexible loop regions, and interestingly, the region of the metalloproteinase domain where the glycosylation sites are found faces the proposed protein–protein adhesive interface of the hyper variable region (HVR) in the cysteine-rich domain (Menezes et al., 2011). In addition, Asn 588 is located very close to the end of the putative HVR of HF3 (Fig. 2). These data led us to hypothesize that an effective protein–protein interaction between the two ligands could be hindered by the presence of the carbohydrate moieties in HF3. Nevertheless, this supposition has to be considered carefully given that DM43 is also glycosylated (León et al., 2012) and HF3 is capable of interacting and cleaving glycoproteins such as fibrinogen, von Willebrand factor and collagen VI (Oliveira et al., 2010).

### 3.2. Effect of α2-macroglobulin on the proteolytic activities of HF3, bothropasin and BJ-PI

Human α2-macroglobulin is a tetrameric protein composed by the noncovalent association of two disulfide-bonded dimers of 360 kDa α2-macroglobulin is a major plasma inhibitor of various classes of proteinases acting by a unique mechanism involving the trapping of the proteinase upon cleavage at its bait region (Borth, 1992). We tested the ability of α2-macroglobulin to prevent the cleavage of fibrinogen and collagen VI by HF3, bothropasin and BJ-PI by preincubating the enzymes with the inhibitor. As shown in Fig. 3A and B, α2-macroglobulin was not effective in inhibiting neither the fibrinogenolytic nor the collagenolytic activities of the proteinases and even failed in affecting the proteolysis by *N*-deglycosylated HF3. The proteinases were also individually incubated with α2-macroglobulin resulting in the degradation of the 180 kDa chain by the proteinases at the following potency order: BJ-PI > bothropasin > HF3 (Fig. 3C). The proteolysis generated a main protein band of ∼90 kDa by the three proteinases. Interestingly, in all incubations of α2-macroglobulin with bothropasin and BJ-PI, 2–3 protein bands of molecular mass higher than 180 kDa appeared on the gels probably resulting from the cleavage of some very high polypeptide chain present in the α2-macroglobulin preparation and that were not visible in the control samples which were not incubated with the proteinases (Fig. 3C).

Furthermore, in order to rule out the possibility that the lack of inhibition by α2-macroglobulin could be caused by its cleavage upon incubation with the proteinases for 30 min, we tested the fast, real time interaction of inhibitor and proteinase by SPR. Injections of native HF3, *N*-deglycosylated HF3, bothropasin and BJ-PI over an α2-macroglobulin sensor chip resulted in no binding (at 0.3 μM) or very low interaction signals (at 1.0 μM) indicating that the association of proteinase and inhibitor is not characterized by a fast association rate (Supplemental Fig. 2).

Earlier studies have demonstrated that α2-macroglobulin is able to inhibit the proteolytic activity of some SVMPs, mainly P-I class enzymes (Rucavado et al., 2005; Baramova et al., 1990; Escalante et al., 2004). Furthermore, the proteolytic activity of jararhagin was reduced when incubated with α2-macroglobulin (Kamiguti et al., 1994); however, no effect on the hemorrhagic activity of this SVMP was observed (Escalante et al., 2003). α2-macroglobulin was also found in greater abundance in the wound exudate and displayed the highest level of

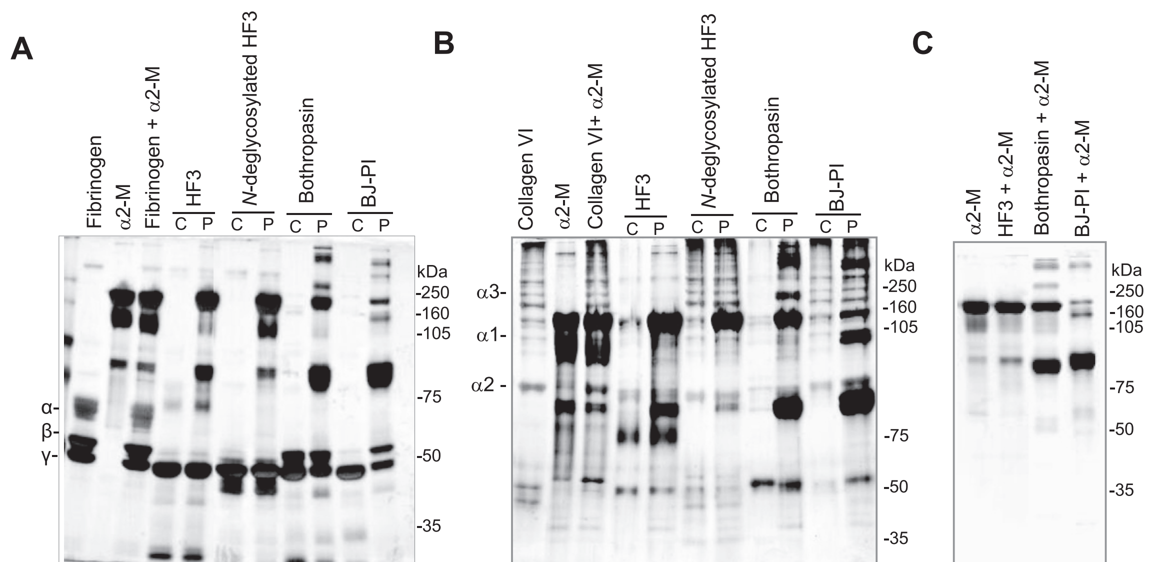


**Fig. 3.** Effect of α2-macroglobulin (α2-M) on the cleavage of fibrinogen and collagen VI by native and *N*-deglycosylated HF3, bothropasin and BJ-PI. The proteinases were preincubated with α2-M and then incubated with human fibrinogen (A) or with collagen VI (B); and α2-M was incubated with the proteinases (C), as described in the Materials and Methods section. (Fibrinogen chains (α, β, and γ) and collagen VI chains (α1, α2 and α3) are indicated. C (control incubation). P (preincubation with α2-M).

degradation among other proteinase inhibitors identified in an investigation of the muscular wound caused in mice by BaP1 (Escalante et al., 2009). Likewise, α2-macroglobulin was found as significantly more abundant in a proteomic analysis of the hemorrhage induced by HF3 in the mouse skin (Paes Leme et al., 2012).

According to the mechanism of inhibition of proteinase activity by α2-macroglobulin, when a proteinase cleaves the inhibitor at its bait region, a conformational change is induced in the inhibitor structure which traps the proteinase and the entrapped proteinase remains active against low molecular mass substrates while the activity against macromolecular substrates is greatly reduced. Moreover, following cleavage in the bait region a thioester bond is hydrolyzed and is involved in the covalent binding of the inhibitor to the proteinase (Barrett and Starkey, 1973). The identification of the sites of specific proteolysis of α2-macroglobulin by atrolysins A, C, D and E showed that the cleavage sites for the four metalloproteinases were within the bait region of the protein (Baramova et al., 1990). Our suggestion for the inability of α2-macroglobulin to inhibit the proteolytic activity of the three proteinases in this study is that the affinity between the ligands (α2-macroglobulin and the proteinases) is very low as no interaction was detected by SPR. Likewise, the cleavage of α2-macroglobulin by HF3, bothropasin and BJ-PI generating a main product of ~90 kDa has not engaged the proteinase in a trapped position required for inhibition by α2-macroglobulin.

### *3.3. Effect of protein DM43 on the hemorrhage induced by HF3 and bothropasin*

To investigate the effects of protein DM43 on the hemorrhage induced by HF3 and bothropasin, mice were injected intradermally on the dorsal region with a fixed dose of HF3 (0.5 μg) and bothropasin (10 μg) preincubated with DM43 (0.7 μg for HF3 and 20 μg for bothropasin). Partial inhibition (43%) of the hemorrhage induced by bothropasin was observed, while HF3 was not affected (Fig. 4) (Table 1). This finding is in agreement with the lack of inhibition of the proteolytic activity of HF3 on fibrinogen and collagen VI by protein DM43, as well as the lack of binding of HF3 to DM43, observed by SPR (Supplemental Fig. 1).

Although the non-catalytic domains of P-III class SVMPs display interesting molecular interactions that may contribute to the efficacy of these enzymes to generate hemorrhage at very low concentration, the catalytic activity of the enzymes is essential for the hemorrhagic process. The susceptibility of P-III class SVMPs to plasma proteinase inhibitors may vary depending on structural features of the proteinases. In the case of MMPs, in the physiological processes in which they participate, the balance between MMP expression, activation and inhibition by TIMPs and other inhibitors is finely regulated (Nagase and Woessner, 1999). In a recent study we showed that some plasma proteinase inhibitors appeared in higher abundance in a proteomic analysis of the skin hemorrhage induced by HF3 (Paes Leme et al., 2012). It is evident that HF3 and bothropasin escape plasma proteinase inhibitors as they were shown to cleave fibrinogen and von Willebrad factor in plasma (Oliveira et al., 2010).

## 4. Conclusions

The first conclusion from the *in vitro* and *in vivo* inhibition assays is that the three proteinases of different domain organization and glycosylation levels were not

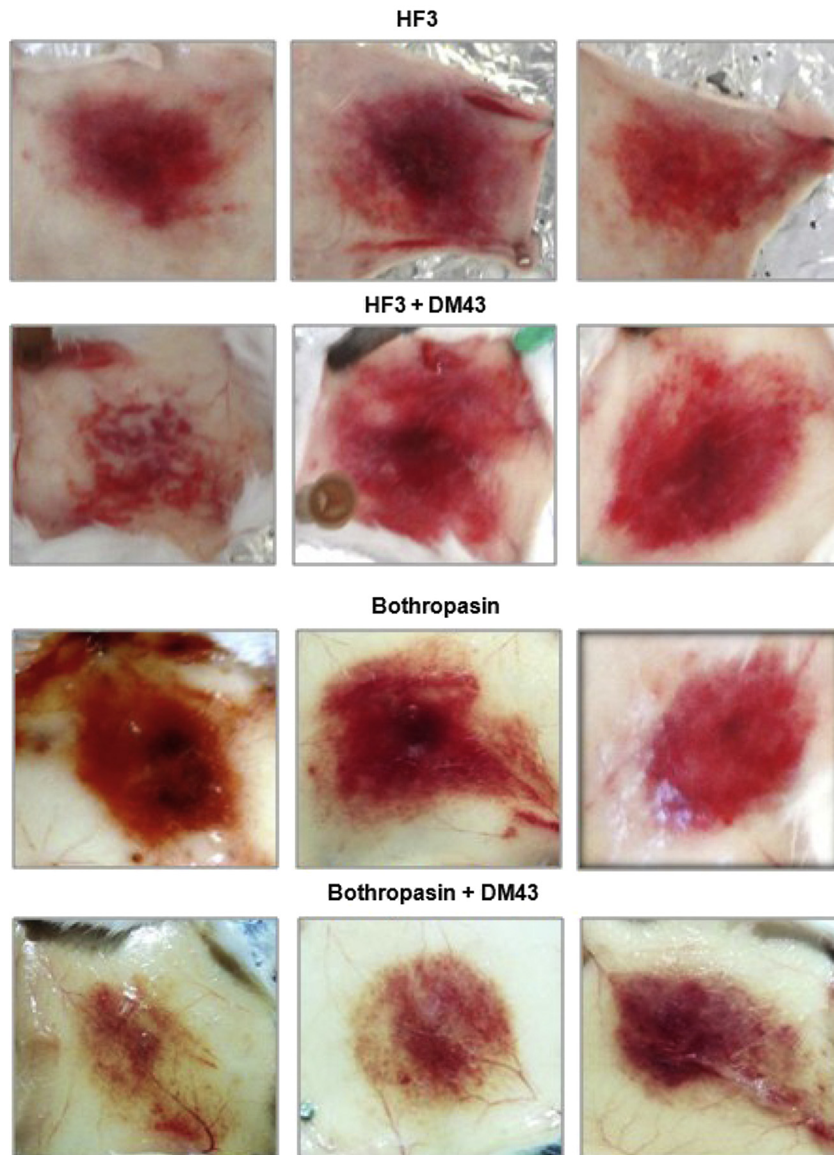


**Fig. 4.** Hemorrhage induced by HF3 and bothropasin on the mouse skin in the absence and presence of protein DM43. Mice were injected intradermally in the dorsal area with HF3 or bothropasin, or with HF3 or bothropasin preincubated with protein DM43, as described in the Materials and Methods section. After 2 h, the skin was removed and the skin underside was photographed.

**Table 1**
Hemorrhagic activity of HF3 and bothropasin in the absence and in the presence of the protein DM43.

| Sample[a] | Hemorrhagic area ($cm^2$) | Inhibition (%) |
|---|---|---|
| 0.5 μg HF3 | 4.2 ± 1.2 | – |
| 0.5 μg HF3 + 0.7 μg DM43 | 5.7 ± 2.4 | None |
| 10 μg bothropasin | 4.0 ± 1.2 | – |
| 10 μg bothropasin +20 μg DM43 | 2.3 ± 0.5 | 43 |

Results are presented as means ± SD ($n = 3$).
[a] HF3 and bothropasin were pre-incubated with the protein DM43 in 50 mM Tris–HCl, pH 8.0 containing 1 mM $CaCl_2$ for 30 min at 37 °C. After 2 h of injection the mouse dorsal skin was sectioned and the underside was photographed.

similarly affected by protein inhibitors and showed rather variable susceptibilities to them depending on the substrate and assay used. Secondly, this investigation emphasizes the role of structural features of the catalytic domains of these proteinases in their interaction with large inhibitor molecules.

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## Conflict of interest statement

The authors declare that there are no conflicts of interest.

## Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.toxicon.2014.01.001.

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